TOXICITY, EXPOSURE, AND RISK OF INSECTICIDES USED FOR MOSQUITO MANAGEMENT ON THE ALFALFA LEAFCUTTING BEE, *MEGACHILE ROTUNDATA*

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Entomology

MONTANA STATE UNIVERSITY
Bozeman, Montana

July 2017

DEDICATION

I would like to dedicate this thesis to my sister, Jenny, whose wedding I nearly missed in order to see this through completion. Thank you for your continued support and for being one of my biggest cheerleaders.

I go down to the shore in the morning
and depending on the hour the waves
are rolling in or moving out,
and I say, oh, I am miserable,
what shall–
what should I do? And the sea says
in its lovely voice:
Excuse me, I have work to do.

-Mary Oliver, *I Go Down to the Shore*

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my major advisor, Dr. Bob Peterson for the continuous support and guidance. You have given me the tools to become successful in the science field and for that, I am eternally grateful for my time spent here at MSU in your lab. Additionally, I thank my committee members, Dr. Michelle Flenniken and Dr. Kevin O'Neill for their support and guidance as well as Dr. Casey Delphia and Ruth O'Neill for their continued support throughout this project. I am grateful to the Mosquito Research Foundation and the Montana Agricultural Experiment Station for funding my graduate studies. Special thanks to the Department of Land Resources and Environmental Sciences for providing a positive learning environment. I would also like to thank past and present members of the CoBRA Lab, especially Dr. Collin Preftakes, Dr. Chris Brown, and Claire Donahoo. I can't thank you enough for the words of encouragement, after work beers, and laughable moments that helped keep me sane throughout this process. I deeply appreciate everything you have helped me with and feel very thankful I was able to work alongside each of you. I would also like to thank my family and friends for all of their support and understanding through this experience; especially Tess, John, and my dog Reed, who were always up for an outdoor adventure when I needed it most, as well as my parents, who always offered words of encouragement. Finally, I would like to thank Jan Hunter, the first person to give me the strength and confidence to pursue this degree. You have taught me so much, but the most important thing was to work hard and love what you do.

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ABSTRACT

The alfalfa leafcutting bee, *Megachile rotundata* F. (Hymenoptera: Megachilidae), is one of the most managed solitary bees and is an important pollinator of many crops, especially alfalfa, *Medicago sativa* L. However, little is known about its response to insecticides, specifically pyrethroids, which are frequently used to manage populations of adult mosquitoes that inhabit the same areas. Current regulatory requirements for insecticide toxicity to non-target insects focus on one pollinator, the honey bee, *Apis mellifera* L., but this species does not represent all insect pollinator species in terms of response to insecticides. Therefore, we characterized the toxicity and risk of three pyrethroid insecticides (permethrin, deltamethrin, and etofenprox) on adult *M. rotundata* in both laboratory and field settings. The median lethal dose, LD_{50}, was estimated for adult *M. rotundata* females when exposed to each pyrethroid to serve as a baseline toxicity test to determine the susceptibility of *M. rotundata* to these insecticides. The range of concentrations for permethrin and etofenprox ranged from 0.0075-0.076 µg/bee and the range for deltamethrin was 0.0014-0.0075 µg/bee. The estimated LD_{50} results for permethrin, etofenprox, and deltamethrin were 0.057, 0.051, and 0.0016 µg/bee, respectively. After obtaining the LD_{50} values, we compared female respiration rates after dosing of each LD_{50} endpoint. In a field study, we applied a formulated version of each active ingredient at the maximum labeled rate of 0.017 kg/ha over an alfalfa field via ultra-low-volume (ULV) applicator and observed mortality of both adult *A. mellifera* and *M. rotundata* for 48-hr after exposure. In both species, there was no significant difference in mortality between control and treated groups for any of the formulations. In another field study, a formulated version of etofenprox was applied in an alfalfa field at the half-maximum labeled rate of 0.003 kg/ha and directly targeted to *M. rotundata* nests. There was no significant difference in mortality between control and treated groups. We also did not observe a significant difference in the number of adults reared between treated vs. control shelters. Results from the field studies suggest that the risk of mortality from these insecticides applied via ULV applicators may be relatively low.

CHAPTER ONE

PROJECT BACKGROUND AND OBJECTIVES

Introduction

Since their first commercial applications in the early 1940s, synthetic organic insecticides have been widely successful in managing many species of pests. The impact of these chemicals, however, may extend beyond the target species to include non-target organisms that occupy the same habitats as pests. One group of insect pests, mosquitoes, are often of high concern due to the number of pathogens they vector. Because of the need to manage mosquitoes on a regular and sometimes intense basis, public concern has focused on the impact these management strategies have on beneficial insect health.

Although many insect species are considered to be beneficial, bees are often used as representative species of non-target organisms, mostly due to human reliance on their pollination services. The U.S. alone produces $15 billion in insect-pollinated crops annually, most of which are pollinated by bees (Kremen et al. 2002). Overall, approximately 30% of the world's crops that are part of the human diet rely on bees for pollination, and as many as 90% of native plant species rely on bees to a greater or lesser extent for reproduction (Pitts-Singer and Cane 2011, Kremen et al. 2002). Although a variety of bees play a crucial role in agriculture and other pollination services, much of the research to date has focused solely on the impacts of insecticides to the honey bee, *Apis mellifera*. While the honey bee is an essential pollinator, they are not the only economically-important pollinator. My research therefore aims to investigate the effects

of three insecticides previously and recently used in mosquito management on the alfalfa leafcutting bee, *Megachile rotundata* (Hymenoptera: Megachilidae), the world's most intensively managed solitary bee (Pitts-Singer and Cane 2011). We chose to specifically research the effects of pyrethroid insecticides as they are one of the most common classes of insecticide used for adult mosquito management. The insecticides chosen represent the three types of synthetic pyrethroids, specifically, permethrin (Type I), deltamethrin (Type II), and etofenprox (non-ester). By observing the effects of these pyrethroids on alfalfa leafcutting bees, better practices can be implemented when managing for adult mosquitoes in both agricultural and residential settings.

Pyrethroid Insecticides

Pyrethroids tend to have low toxicity to terrestrial vertebrates and break down rapidly in the environment, but are wide-ranging insecticides used to control mosquitoes in many areas of the U.S. and are toxic to all insects (Jensen et al. 1999). Targeted adulticide applications create aerosolized clouds of insecticide that contact and kill flying adult mosquitoes immediately after application (Carney et al. 2008). Although effective, public concern about insecticides has increased due to the potential impact on non-target organisms (Jensen et al. 1999, Their 2001).

The use of pyrethroid insecticides has increased greatly within the past few decades. The introduction of permethrin in 1973 made it one of the most widely used pyrethroids in the U.S. due to its photostability compared to previous formulations offered (USEPA 2015a, Elliott et al. 1973, Schleier III and Peterson 2011). Today,

pyrethroids are estimated to comprise 23% of the world's insecticide market and are used in both agricultural and residential areas across the U.S. (Schleier III and Peterson 2011). For mosquito control, these chemicals are most often used to manage adult females using aerial and ground applications of ultra-low-volume (ULV) pyrethroids (Roche 2002).

Pyrethroids are synthetic byproducts of pyrethrums, which are a combination of six essential oils extracted from chrysanthemum flowers (USEPA 2015a). These extracts are known to be one of the oldest and widely used botanical insecticides, dating back to early Native American and Chinese culture. Synthetic pyrethroids were introduced as a substitution for pyrethrums, as pyrethrums tend to have low photostability and are relatively expensive to produce when compared to synthetics. Similarly, synthetic pyrethroids tend to be non-bioaccumulative within the ecosystem, quickly knock down insects within the targeted area, and have low mammalian toxicity (Swain et al. 2009, Schleier III and Peterson 2011).

The three classes of pyrethroids, Type I, Type II, and non-ester, all affect insect nervous systems by modifying the voltage-gated sodium channels (VGSC) in different ways. Each type of pyrethroid cause the nervous system to release neurotransmitters, which cause multiple actions within insects. Most control over bodily functions are disoriented via loss of coordination, convulsive body movements, or paralysis, often killing organisms quickly when they are exposed directly (Schleier III and Peterson 2012). However, there are toxicological variations among the three classes of pyrethroids. Effects of Type I pyrethroids include induction of whole body tremors (T syndrome), sensitivity to external stimuli, and the rise in core body temperature due to excessive

muscle tremors. Type I pyrethroids, such as permethrin, act on sodium channels while they are closed. By comparison, effects of Type II pyrethroids, such as deltamethrin, are characterized by CS syndrome, or sinuous and writhing movements that causes a decrease in the core body temperature and alters sodium channels while they are open and inactive (Schleier III and Peterson 2011, Schleier III and Peterson 2012). Similar to Type I pyrethroids, non-esters (e.g., etofenprox) prolong the open time of VGSCs in nerve cell axons, inducing repetitive firing of these VGSCs and in turn affecting repetitive activity in the sensory and motor pathways (Soderlund 2010, Schleier III and Peterson 2012, Hoang et al. 2010).

Exposure of Non-Target Insects to ULV Insecticides

Ground-based applications of aerosols of various insecticides have been used to effectively control mosquito populations for a number of years. Since the introduction of ultra-low-volume (ULV) technology, a variety of methods have been used to better understand effectiveness and decrease environmental contamination (Lofgren et al. 1973).

ULV applications have very small droplet sizes ranging from 8 to 30 μm, which are the optimum size to impinge on flying adult mosquitoes (Lofgren et al. 1973, Schleier III et al. 2012). Droplets this small have a large surface area available to contact adult flying mosquitoes and are effective at contacting target mosquitoes and are quickly absorbed through the cuticle. ULV has therefore become a popular management option for adult mosquitoes because it has been shown to reduce mosquito populations and

reduce disease infection rates of mosquito hosts (Carney et al. 2008). Oil and water are two common additives that aid in dispersal of insecticides for ULV application. The varying densities of these two additives can alter the movement and deposition of the insecticide, resulting in different levels of exposure (Preftakes et al. 2011).

Sprayed insecticide applications via ULV exposure tends to stay aloft in the air and results in very low percentages of insecticide deposited on leaf surfaces and surrounding environments, with only 1 to 30% of insecticides being deposited within the spray swath (Lofgren et al. 1973, Knepper et al. 1996, Schleier III et al. 2012). Agricultural applications have been designed to minimize droplet movement so that residues are equally distributed through varying heights in the swath width (Schleier III and Peterson 2010, Preftakes et al. 2011). Several studies have indicated that daytime application of pyrethroids where honey bees were placed in cages in direct line with insecticide drift resulted in high population mortality (Caron 1979, Hester et al. 2006). However, Caron (1979) also found that night applications had little to no mortality on bee populations. Similarly, Jensen et al. (1999) found that ground application of permethrin and malathion highly affected insects flying at night, but populations became abundant once more 48 hours following application. A study by Davis and Peterson (2008) found that multiple ULV applications provided minimal risk to ecosystem dynamics for arthropod species in terrestrial and aquatic environments. However, recent studies have suggested that when non-target organisms are protected from direct contact with the aerosol clouds of insecticide (i.e., by vegetative covering), especially at increasing distances from the spray source, non-target mortality can be mitigated (Rinkevich et al.

2017, Peterson et al. 2016). Similarly, ULV applications used for adult mosquito management are most effective when the insecticide remains airborne and moves through the target area (Schleier III et al. 2012). Although the impacts of adulticide tend to be low to organisms that are inactive during the evening hours, deposited residue of pyrethroids on flowers, leaves, and nests could have negative effects on populations of *M. rotundata*, as pyrethroids are highly toxic to bee species.

The Shift from One Surrogate Species to Another

Under the Federal Insecticide Fungicide and Rodenticide Act (FIRFA), the U.S. EPA lists the honey bee as the standard non-target insect species required for testing by chemical registrants in the U.S. (Hoang et al. 2010). Although honey bees are important non-target organisms, they should not be the only surrogate species used in testing. Due to major differences in physiology and morphology between this species and other taxa of prolific pollinators, honey bees should not be solely used to determine insecticide impacts of other beneficial insects (Hoang et al. 2010).

The main differences in insecticide sensitivity among species are in absorption, distribution, metabolism, excretion, and body mass (Thompson 2015). As an example, *A. mellifera* workers weigh approximately 0.10 g, while *M. rotundata* females average 0.03 g (Pitts-Singer and Cane 2011). Additionally, *M. rotundata* contact insecticide-contaminated foliage in ways that are different from the contact *A. mellifera* exhibits. As discussed below, alfalfa leafcutting bees cut sections of alfalfa foliage to line nest cells. The high sensitivity of *A. mellifera* is potentially confirmed by the lower number of genes

encoding xenobiotic detoxifying enzymes within its genome compared to other insect species, specifically other Apiformes. This deficit of detoxification genes may not be exclusive to *A. mellifera*, but may be a specific adaptation within the eusocial insect group (Claudianos et al. 2006, Hardstone and Scott 2010, Arena and Sgolastra 2014). Because of this specific adaptation, the varying patterns of different social behaviors are therefore important to pollinators' response to insecticides (Arena and Sgolastra 2014). Therefore, the responses of one surrogate species do not provide sufficient information to characterize pollinators as a whole and a variety of different species should be observed in order to better understand the potential risk insecticides pose on pollinator species.

Why leafcutting bees?

Examining *M. rotundata* susceptibility is just one step in providing detailed information on how the array of pollinators that humans rely on respond to insecticide application in both agricultural and residential areas. *Megachile rotundata* was accidentally introduced to the U.S. in the 1940s from Europe and Asia and then subsequently used boost the alfalfa seed industry because honey bees and native pollinators could not keep up with the increase in demand (Thompson 2015). One of the major problems with using *A. mellifera* for alfalfa pollination is that it has adapted to forage for nectar without tripping the alfalfa flowers, circumventing the pollination mechanism (Baird et al. 1991). *A. mellifera* often circumvents cross-pollination of alfalfa and chews a hole in the corolla to easy extract nectar. In addition, alfalfa pollen is less attractive to them compared to some other plant species primarily because the stamens

tend to forcefully strike them in the head due to their larger body mass. (Stephen 1965, Pitts-Singer and Bosch 2010). Because of these unfavorable conditions, *A. mellifera* often circumvents cross-pollination of *M. sativa* and chews a hole in the corolla to easy extract nectar. Hybrids of *M. sativa* have been produced, but breeding ideal flowers for *A. mellifera* can be both time consuming and expensive.

Alfalfa leafcutting bees, on the other hand, have demonstrated many desirable traits for alfalfa pollination. Once established, *M. rotundata* increased seed production of several different plant species in the family Fabaceae by three times the previous amount (Scott-Dupree et al. 1995). Typically, *M. rotundata* is capable of tripping the staminal column of alfalfa up to 80% of the time when flowers are visited (Pitts-Singer and Cane 2011), far exceeding the successful trip rate of honey bees. This tripping of the flower releases pollen on the scopa of female *M. rotundata* and allows the female to extract nectar from the plant, inducing cross-pollination and benefiting female *M. rotundata* (Scott-Dupree et al. 1995, O'Neill 2004).

The productivity of *M. rotundata* has become an essential part of the alfalfa seed industry primarily because of our dependence of alfalfa as a primary source of cattle feed. Alfalfa hay is sold worldwide as food for cattle at an annual rate of $4.6 billion per year; one-third of the $15 billion revenue credited to honey bees pollinating U.S. crops (Pitts-Singer and Cane 2011). The combination of the *M. rotundata* life cycle coinciding with peak alfalfa bloom as well as advantageous morphological characteristics has resulted in *M. rotundata* becoming the most efficient pollinator of alfalfa and one of the most

intensively managed solitary bees (Pitts-Singer and Cane 2011, Pitts-Singer and Bosch 2010, O'Neill 2004).

The Life Cycle of *Megachile rotundata*

Unlike honey bees and bumble bees (*Bombus* spp.), alfalfa leafcutting bees do not continuously produce new generations of foragers throughout the growing season. Instead, they synchronize their emergence with the peak bloom of alfalfa, typically a four to six-week period and offspring do not emerge until the following growing season (O'Neill et al. 2015). As soon as females begin to emerge from their nest cells, they begin to mate with already emerged males. Male *M. rotundata* emerge three to five days before to females, and perch near nest entrances or on nearby flora to mate with newly eclosed females (Pitts-Singer and Bosh 2010). Females typically mate only once and can lay as many as two eggs per day, totaling an average of 57 eggs in their two-month adult life span, depending on the amount floral resources (Klostermeyer and Gerber 1969, Pitts-Singer and Cane 2011). Farmers using alfalfa leafcutting bees for alfalfa production need to carefully estimate acreage and harvesting potential, as an influx of bees could be economically wasteful and result in nutritionally depleted offspring for the next growing season (Pitts-Singer and Bosch 2010, O'Neill et al. 2011). On average, approximately 20,000 to 30,000 female bees per acre (or 50,000 to 75,000 per hectare) are needed to pollinate the alfalfa crop (Scott-Dupree et al. 1995).

Mating of *M. rotundata* is usually seen at commercial nesting shelters. Because *M. rotundata* are highly gregarious, these shelters are comprised of thousands of small cavities in which females provision nests. Females use olfactory stimuli to navigate to

and from their own nests sites (Guedot et al. 2013). These shelters vary in size depending on the acreage of the farm, and are placed in the field before adults have emerged.

Females lay each egg in its own cell (typically comprised of 14-15 leaf pieces) on top of a provision mixture of 33-36% pollen and 64-67% nectar, decreasing in pollen and increasing in nectar as the season progresses. Larger provision masses are laid first in nest tubes and are reserved for female offspring (Klostermeyer and Gerber 1969, Pitts-Singer and Bosh 2010). Males will typically receive provision masses that weigh 17% less than those provided for females, resulting in smaller male body size and quicker maturation, and are laid near the entrance of the cell.

Following egg hatching, larvae eat the entire pollen provision (typically leaving less than 2% of the provision), and then overwinter as a diapausing larvae. Diapause ceases in the early spring or summer as environmental temperature begins to rise and adults emerge.

On a commercial level, a common practice for collection and storage is called "loose cell bee management system" (Scott-Dupree 1995, Pitts-Singer and Cane 2011) where nest boxes are collected from the field; cells are removed from nesting boards, cleaned, and then stored for the winter. Cells are typically stored in a cold environment around 4°C for no less than 6 months. Then, just before initial adult male emergence, the cells are placed back in the field near the nest shelters. Loose cell management is commonly used as it allows for easier management of adverse conditions such as chalkbrood and parasitism (O'Neill 2004).

Objectives and Rationale

To gain a more complete understanding of the effects of pyrethroids on the alfalfa leafcutting bee, various routes of exposure occurring at different stages of the life cycle need to be examined. The purpose of our research will therefore be to establish base-line toxicity levels of three pyrethroids as well as simulate two worst-case scenario settings within two field studies. We aim to address these research topics with three objectives:

Objective 1: Estimate the LD_{50} values and respiratory patterns of *M. rotundata* females after exposure for permethrin, deltamethrin and etofenprox.

Objective 2: Evaluate mortality of female *M. rotundata* and *A. mellifera* after contact with alfalfa foliage exposed to Aqua-Reslin® (permethrin), DeltaGard® (deltamethrin), and Zenivex® E20 (etofenprox) via ULV applicator.

Objective 3: Determine the effects of Zenivex® E20 on nesting *M. rotundata* adults and next generation larvae and adults.

CHAPTER TWO

ACUTE TOXICITY OF PERMETHRIN, DELTAMETHRIN, AND ETOFENPROX TO THE ALFALFA LEAFCUTTING BEE, *MEGACHILE ROTUNDATA* (HYMENOPTERA: MEGACHILIDAE)

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Manuscript Information Page

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Status of Manuscript:
_X_ Prepared for submission to a peer-reviewed journal
____ Officially submitted to a peer-review journal
____ Accepted by a peer-reviewed journal
____ Published in a peer-reviewed journal

CHAPTER TWO

ACUTE TOXICITY OF PERMETHRIN, DELTAMETHRIN, AND ETOFENPROX TO THE ALFALFA LEAFCUTTING BEE, *MEGACHILE ROTUNDATA* (HYMENOPTERA: MEGACHILIDAE)

The following chapter has been prepared for submission to a peer-reviewed journal

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Abstract

Current regulatory requirements for insecticide toxicity to non-target insects focus on the honey bee, *Apis mellifera* L., but this species cannot represent all insect pollinator species in terms of response to insecticides. Therefore, we characterized the toxicity of pyrethroid insecticides used for adult mosquito management (permethrin, deltamethrin, and etofenprox) on a non-target insect, the adult alfalfa leafcutting bee, *Megachile rotundata* F. The doses causing 50% and 90% mortality (LD_{50} and LD_{90}, respectively) were used as endpoints in this study. Two-day-old adult females were exposed to eight concentrations ranging from 0.0075-0.076 μg/bee for permethrin and etofenprox, and 0.0013-0.0075 μg/bee for deltamethrin. Respiration rates of female *M. rotundata* were also recorded for two hours after bees were dosed at the LD_{50} values to give an indication

of stress. Results indicated a similar LD_{50} for permethrin and etofenprox, 0.057 and 0.051 μg/bee, respectively, and a more toxic response, 0.0016 μg/bee for deltamethrin. Comparatively, female *Apis mellifera* workers have a LD_{50} value of 0.024 μg/bee for permethrin and 0.015 μg/bee for etofenprox indicating that female *M. rotundata* are less susceptible to topical doses of these insecticides, except for deltamethrin, where both *A. mellifera* and *M. rotundata* have an identical LD_{50} of 0.0016 μg/bee. Respiration rates comparing each active ingredient to control groups, as well as rates between each active ingredient, were statistically different ($P < 0.0001$). The addition of these results to existing information on *A. mellifera* may provide more insights on how other beneficial, non-target bees respond to pyrethroids.

Introduction

Since the accidental introduction of the alfalfa leafcutting bee, *Megachile rotundata* F. (Hymenoptera: Megachilidae), from Eurasia to the U.S. in 1940, *M. rotundata* has aided in the $4.6 billion per year alfalfa hay industry, through its role in alfalfa seed production (Pitts-Singer and Cane 2011). The $4.6 billion value is one-third of the $14 billion value credited to honey bees, *Apis mellifera* L., pollinating U.S. crops (Pitts-Singer and Bosch 2010), which means *M. rotundata* is an important component of the U.S. agricultural industry.

Wild and managed solitary bee species play a crucial role in the pollination of many crops in the U.S. and worldwide. However, the potential risks of insecticides are not widely known for many pollinators, other than the honey bee (Artz and Pitts-Singer

2015, Pitts-Singer and Cane 2011). *Megachile rotundata* is the second most intensively managed pollinator in the U.S., but little is known about its susceptibility to different insecticide classes. Although most research primarily focuses on lethal doses affecting *A. mellifera*, this might not be the best surrogate species for all bees because its life history is different from that of other pollinators and because it may have different toxic endpoints than other species in the same or different families. Differences in their responses to toxin would not be surprising, given that the last common ancestor of *A. mellifera* and *M. rotundata* is typically estimated to have lived over 100 million years ago (Timetree of Life 2017).

Following the introduction of permethrin in 1973, the use of pyrethroids in the U.S. has dramatically increased. Pyrethroids currently account for approximately 23% of the world's insecticide market and are used in both agricultural and residential areas, particularly to manage adult mosquitoes (Elliott et al. 1973, Schleier III and Peterson 2011). As diseases such as West Nile virus, chikungunya, dengue, and Zika continue to circulate, vector management practices will continue to be intensively and extensively implemented. With these practices, there may be adverse effects on non-target beneficial organisms, specifically pollinators (Davis et al. 2007).

One of the first steps in addressing these potential effects is by identifying the acute lethal dose of insecticides to the organism of interest. Therefore, the goal of this study was to estimate the acute toxicity of three types of pyrethroids (permethrin, deltamethrin, and etofenprox) to adult female *M. rotundata*. The pyrethroids we evaluated not only represent Type I, Type II, and non-ester pyrethroids, respectively, but

they also are used in adult mosquito management. In addition, we measured respiration rates to assess how these insecticides affect *M. rotundata* after exposure. We chose to measure respiration rates after exposure to each of the active ingredients because insect respiration is often used as an index of stress (Kestler 1991).

Materials and Methods

Insects

Diapausing *M. rotundata* larvae in loose nest cells were purchased from JWM Leafcutters, Inc. (Nampa, Idaho) in April 2015 and 2016 and were placed in room temperature (23°C) for three days before being placed in the rearing room set to 28 ± 2°C, relative humidity 42-60%, and a photoperiod of 16:8 (L:D) hours; post-diapause rearing of *M. rotundata* at 28°C results in high emergence rates and adults with high lipid content (Baird and Bitner 1991, O'Neill et al. 2011). Cells of *M. rotundata* were placed inside Specimen Transfer Cages No-See-Um Mesh, 61 x 61 cm (BioQuip Products, Inc., Rancho Dominguez, California) and reared for 15-25 days. Each day, as adults emerged, they were removed from the cage using an aspirator and then transferred to a refrigerator set at 4°C for approximately 20 min. Once the bees were immobile, they were removed from the container and sorted by sex on a Laboratory Chill Table (BioQuip Products, Inc., Rancho Dominguez, California) and females were collected to perform the assay. After females were sorted from males, 10 females were placed in 20 mL glass Wheaton™ scintillation vials (Thermo Fisher Scientific Co., Waltham, Massachusetts) and stored briefly until dosing on the same day that they emerged or the following day.

Chemicals

The insecticides permethrin ((3-phenoxyphenyl)methyl 3-(2,2-dichloroethenyl)-2,2dimethylcyclopropane carboxylate), etofenprox (2-(4-ethoxyphenyl)-2-methylpropyl 3-phenoxybenzyl-ether), and deltamethrin ((S)-α-cyano-3-phenoxybenzyl (1R,3R)-3-(2,2- dibromovinyl)-2,2-dimethylcyclopropane carboxylate) were used as the active ingredients for this assay because they are used in mosquito management and they represent the three types of synthetic pyrethroids: Type I (permethrin), Type II etofenprox), and non-ester (deltamethrin) (USEPA 2009). Technical grade permethrin, etofenprox, and deltamethrin of 98% purity was purchased from Sigma-Aldrich (St. Louis, Missouri) and stock solutions were prepared in acetone (99.7% purity) purchased from EMD Chemical (Gibsontown, New Jersey) (USEPA 2012a).

LD_{50} Bioassay

The U.S. Environmental Protection Agency (USEPA) protocol and previous experiments in our laboratory for LD_{50} dosing was followed for this assay (USEPA 2012a, Whiten and Peterson 2015). The purpose of this test was to determine the quantity of test substance that causes 50% mortality in the test population of female *M. rotundata*. Females were randomly assigned to alternative dose levels or control group and each female received a 2 μL topical dose (active ingredient plus solvent) on the dorsum of the thorax via Eppendorf Reference® micropipette (Eppendorf AG, Hamburg, Germany).

Ninety female bees were exposed to the test substance for each replication. For each insecticide, ten female bees were assigned to each of eight dose concentrations plus

an acetone-only control. Dose concentrations for permethrin and etofenprox were the same because they pose similar toxicity (Deo et al. 1988), while we used a lower range of deltamethrin concentrations (USEPA 1992) because it is much more toxic to invertebrates. For permethrin and etofenprox, the concentrations were 0.0075, 0.0225, 0.0375, 0.0450, 0.0525, 0.0600, 0.0675, and 0.0760 μg/bee. For deltamethrin, the concentrations were 0.0013, 0.0019, 0.0026, 0.0030, 0.0040, 0.0050, 0.0060, and 0.0075 μg/bee, where each female wet weight was approximately 0.035 g . For each concentration, chilled, immobile females were topically dosed to their randomly assigned concentration on a wooden Adjustable Spreading Board (BioQuip Products, Inc., Rancho Dominguez, California) covered with SaranWrap™ (SC Johnson Brands, Racine, Wisconsin) and replaced for each concentration.

After females were dosed, they were placed into 500-mL Tupperware® (Tupperware Brands Corporation, Orlando, Florida) with 36 holes covering the entire lid, for 24 hours and stored at room temperature (27 ± 5 °C). All bees were then observed for 24 hours after treatment and were observed for mortality at the end of this time period. A bee was considered dead when completely immobile and unresponsive to probing.

Respiration

A closed-system respirometer (LI-COR Li-6400XT, LiCor, Lincoln, Nebraska) was used to measure the CO_2 of dosed *M. rotundata* females. As in the LD_{50} assay, females were dosed on the dorsum of the thorax with 2 μL of permethrin, etofenprox, or deltamethrin (active ingredient plus solvent) at the estimated LD_{50} value, or were dosed with 2 μL of acetone as a control. Ten female bees (0.35 g, total wet weight, 0.035 g per

individual female) were placed in a cylindrical chamber 10-cm long x 3-cm diameter (Li-6400XT, 6400-89 Insect Respiration Chamber) for 2 hr. The chamber was darkened by covering it with a thick sheet of paper for the duration of the experiment to ensure minimal visual stimulation of the bees. Ten bees were placed within the chamber to ensure a sufficient level of respiration could be recorded. The cylindrical chamber was connected to a gas analyzer that compared the air from the respiration chamber to a standard sample of 380 μmol CO_2 and measured the flux CO_2 rate of the chamber every 30 sec for 2 hr. Because we only had one Li-6400 unit, the order of treatments was randomized as paired treatments (i.e., permethrin/control, etofenprox/control, or deltamethrin/control). The paired (control and treated) measurements were replicated four times for the three active ingredients + control (10 bees/treatment/replication, n = 360). Respiration rates were recorded every 30 sec for two hours for each group. Recordings were then averaged over a 5-min period resulting in 182 respiration outputs for each of the treatment and control groups. We then log-transformed the respiration values to achieve normal distribution of the data.

Statistical Analysis

For the LD_{50} analysis, experiments for each active ingredient + control were replicated a total of seven times (90 organisms per replication, n = 630) in the summer of 2015 and 2016. Treatment mortality was corrected using Abbott's formula (Abbott 1925) and replications were not used in the analysis if mortality was greater than 20% in the control groups (Yu 2008). For each concentration, the data were analyzed by probit

analysis using Polo Plus (LeOrca 2002). The LD_{50} and LD_{90} and associated 95% confidence intervals were calculated for each active ingredient.

To characterize respiration rates for female *M. rotundata* exposed to the three active ingredients at the LD_{50} level, we analyzed the difference in intercepts of the respiration rates of 10 females a total of four times for each active ingredient and control using a two-way ANOVA linear regression model ($\alpha = 0.05$) (R Studio, Inc. 1.0.136). Similar statistical measures were used to compare the differences of each active ingredient from each another (n = 280). Groups were recorded over a period of two hours and readings were recorded every 30 sec for that time period. Recordings were then averaged over a 5-min period resulting in 182 respiration outputs for each of the treatment and control groups. We then log-transformed the respiration values to achieve normal distribution of the data.

Results and Discussion

The LD_{50} for adult, 2-day-old female *M. rotundata* was estimated for permethrin, etofenprox, and deltamethrin. The estimated LD_{50} for each of the active ingredients was 0.057 µg/bee (95% CI = 0.05-0.08) for permethrin, 0.051 µg/bee for etofenprox, and 0.0016 µg/bee (95% CI = 0.0014-0.0018) for deltamethrin As expected, when comparing the LD_{50} values across active ingredients (Table 1), the most toxic active ingredient for 2-day old female *M. rotundata* was deltamethrin whose confidence intervals did not overlap those of the other two insecticides. Estimated LD_{50} values *for A. mellifera* (0.0016 µg/bee) and *M. rotundata* were identical for this active ingredient (USEPA

1992). However, although *A. mellifera* has LD_{50} values of 0.024 μg/bee for permethrin and 0.015 μg/bee for etofenprox (USEPA 1992), our results indicate that *M. rotundata* would be more tolerant to these active ingredients. Thus, deltamethrin is more toxic to *M. rotundata* compared to permethrin and etofenprox, but *A. mellifera* is likely more susceptible to these active ingredients compared to *M. rotundata,* and all three active ingredients are highly toxic to both bee species based on standards set by Felton et al. (1986) (i.e., they have LD_{50} values <1.0 μg/bee).

The estimated difference in respiration between permethrin and the control group was 13.60 μg CO_2/g insect/min (n = 4, df= 189, SE = 2.08, P < 0.0001) (Fig. 1). The difference between deltamethrin and the control was 10.09 μg CO_2/g insect/min (n = 4, df = 189, SE = 2.06, P < 0.0001) (Fig. 2). Etofenprox had the largest mean difference in respiration between active ingredient and control group (55.83 μg CO_2/g insect/min) (n = 4, df = 189, SE = 3.36, P < 0.0001) (Fig. 3). Similarly, when respiration rates were compared to each other, they were statistically different from one another (P < 0.001) (n = 3, df = 278, SE = 3.30), indicating that these pyrethroids may alter the respiration of *M. rotundata* in different degrees and ways (Fig. 4).

The decrease in respiration rates for both the control and treated groups during the 120 min they were in the chamber can be at least partly explained by the excited state of the bees when they were first placed in the test chamber. Following the same experimental design from the LD_{50} assay, females were cooled before dosing to ensure they were immobile during the topical application of each active ingredient. We therefore waited approximately 5-10 min before the initial recording in the Li-Cor insect chamber

and started recording when all test subjects were mobile. This method ensured that the bees were both active during the initial recording and that we were capturing the initial response to each treatment. We assumed that the bees would exhibit high activity at the start of recording due to continuous movement throughout the insect chamber, but lower activity as time progresses and they became used to being in the chamber with other bees, based on behavior we observed while working with them. Although this movement affected the respiration recordings, it occurred in both control and treated groups and there was a significant difference between the groups treated with each active ingredient and the controls. Despite a paucity of reported data on insect respiration rates, especially with hymenopteran species, our rates were similar to those previously reported (Contreras and Bradley 2009, Marias et al. 2005).

The trend in toxicity across the three types of pyrethroids we tested on the same species when topically dosed is not surprising. All pyrethroids disrupt normal cellular communication by modifying the kinetics of voltage-gated sodium channels (VGSCs) (Soderlund 2010). Type I (permethrin) and non-ester (etofenprox) pyrethroids affect the insect nervous system in a similar manner, but the effects of Type II (deltamethrin) pyrethroids may differ considerably from the other two types. Type I and non-ester pyrethroids prolong the openings of VGSCs during the closed state, whereas Type II pyrethroids cause depolarization of the membrane, resulting in increased resting membrane potential (Schleier III and Peterson 2011). Although VGSCs in insects may be affected differently, all pyrethroids affect the nervous system in insects by resulting in a loss of coordinated movements, periods of convulsive activity, and/or terminal paralysis

(Soderlund and Bloomquist 1989, Schleier III and Peterson 2011, Schleier III and Peterson 2012).

Although the effect on VGSCs in insects is known, the effect that insecticides have on targeted or non-targeted organisms is complex and can vary depending on the species. Even though we saw similar trends in *M. rotundata* compared to published values for *A. mellifera* (USEPA 1992), the toxic endpoints varied, and not necessarily in ways we would expect. Adult workers of *A. mellifera* (0.10 g wet weight) (Aronstein et al. 2012) are approximately three times the size of adult female *M. rotundata* (0.03 g wet weight), yet *A. mellifera* tends to be more susceptible to these active ingredients (Hardstone and Scott 2010) compared to *M. rotundata*, based on our findings compared to studies reported in the literature (USEPA 1992). It should be noted that the LD_{50} of certain organisms can vary depending on many variables, including, but not limited to, the experimental protocol used, the overall health of the population of test species, age of the test species, or mode of exposure. This reiterates the idea that the main differences in insecticide sensitivity among species can include several factors such as absorption, internal and external distribution, excretion, and body mass (Thompson 2015).

Claudianos et al. (2006) compared the genome of *A. mellifera* to *Drosophila melanogaster* and *Anopheles gambiae* and found that *A. mellifera* contained significantly less annotated genes than the two dipteran species, specifically, cytochrome P450. The cytochrome P450 enzymes function to aid in metabolizing toxic compounds, including pyrethroids (Claudianos et al. 2006, Yu et al. 1984). Therefore, if *M. rotundata* has

significantly more cytochrome P450 enzymes than *A. mellifera* this may explain the differences in acute toxicity between the two species.

Under the Federal Insecticide and Rodenticide Act (FIRFA), the U.S. EPA lists *A. mellifera* as the standard non-target insect species required for testing by chemical registrants in the U.S. (Hoang et al. 2010). Although information for *A. mellifera* is necessary, more information is needed to better understand insecticide risk to non-target organisms. One species does not necessarily provide adequate information to characterize the risk insecticides posed to hymenopteran pollinators. Toxicities can vary depending on the specific insecticide and species tested, and economically important species should be tested (Hardstone and Scott 2010). Therefore, data obtained from *M. rotundata* will augment existing data related to risks of insecticide application.

Because acute LD_{50} values for *A. mellifera* for permethrin and etofenprox are lower than the values for *M. rotundata*, it may be possible to use *A. mellifera* as a conservative non-target surrogate for *M. rotundata*. Furthermore, this might be possible not only for permethrin and etofenprox, but also more broadly for Type I and non-ester pyrethroids. Moreover, if *A. mellifera* is more susceptible to these pyrethroids than some solitary bee species of such genera as *Osmia*, *Nomia*, *Lasioglossum*, *Colletes*, and *Halictus*, then it might be possible to use it as a conservative non-target surrogate. However, broader testing of multiple bee taxa in diverse evolutionary lineages will be necessary to establish this.

Acknowledgments

We thank the members of the CoBRA Lab: C. Donahoo for assistance in data collection, C. Preftakes for assistance with statistical analysis, and M. Cochran for initial LD_{50} lab work. We also thank JWM Leafcutters, Inc. for providing the test bees. This research was funded by the Mosquito Research Foundation, the Montana Agricultural Experiment Station, and Montana State University. This material is based on work that is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, Hatch Multistate Project No. W-3045.

Table 1. Estimated LD_{50} and LD_{90} values for *Megachile rotundata*

Active Ingredient	n	Slope (SE)	LD_{50} (µg/bee)	95% CI (µg/bee)	LD_{90} (µg/bee)	95% CI (µg/bee)
Permethrin	630	1.66 (0.19)	0.057	0.05-0.08	0.337	0.22-1.30
Etofenprox	630	2.43 (0.26)	0.051	0.04-0.06	0.173	0.12-0.33
Deltamethrin	630	4.26 (0.44)	0.0016	0.0014-0.0018	0.0052	0.004-0.0078

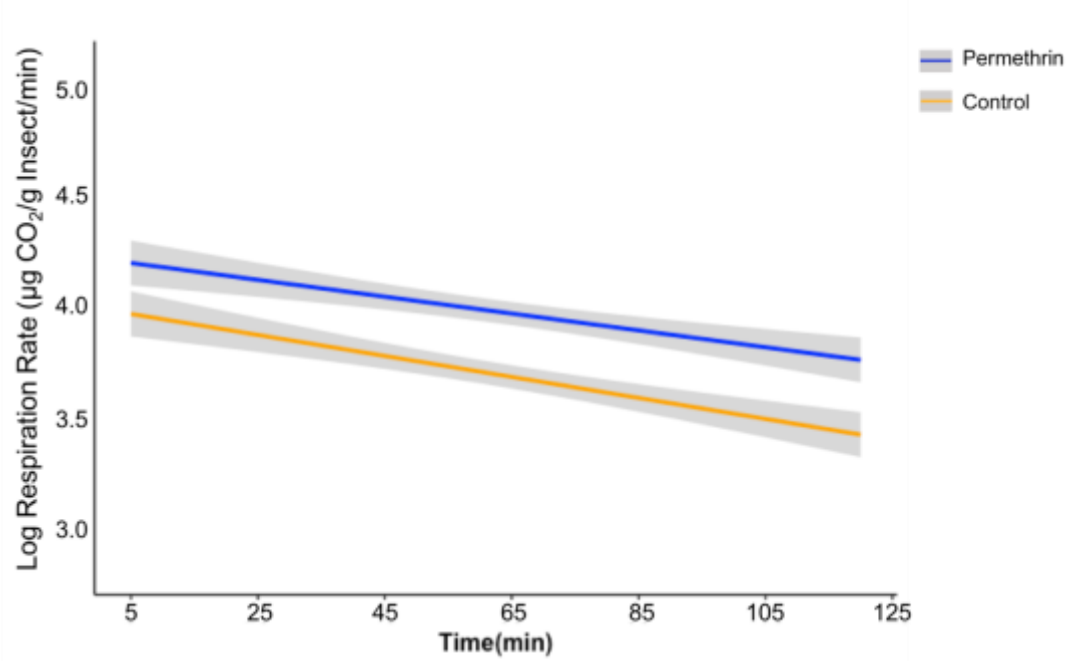

Figure 1. Log changes in respiration as a function of time after dosing at the LD_{50} value for permethrin (blue) compared to the control, acetone (orange). Confidence intervals are represented in the gray-shaded region of each line.

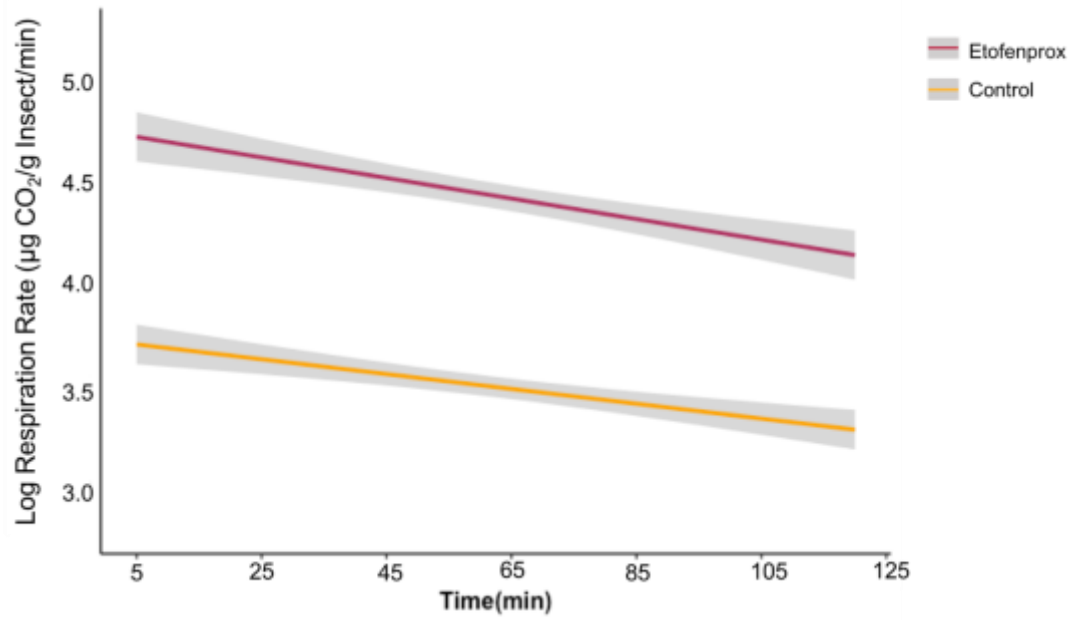

Figure 2. Log changes in respiration as a function of time after dosing at the LD_{50} value for etofenprox (red) compared to the control, acetone (orange). Confidence intervals are represented in the gray-shaded region of each line.

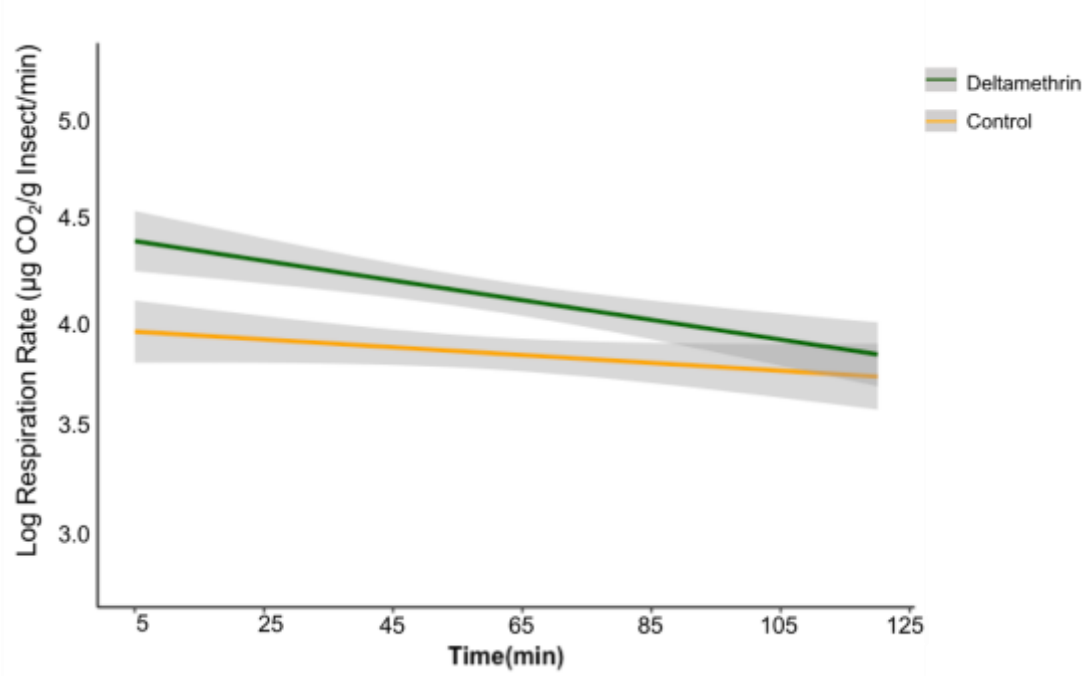

Figure 3. Log changes in respiration as a function of time after dosing at the LD_{50} value for deltamethrin (green) compared to the control, acetone (orange). Confidence intervals are represented in the gray-shaded region of each line.

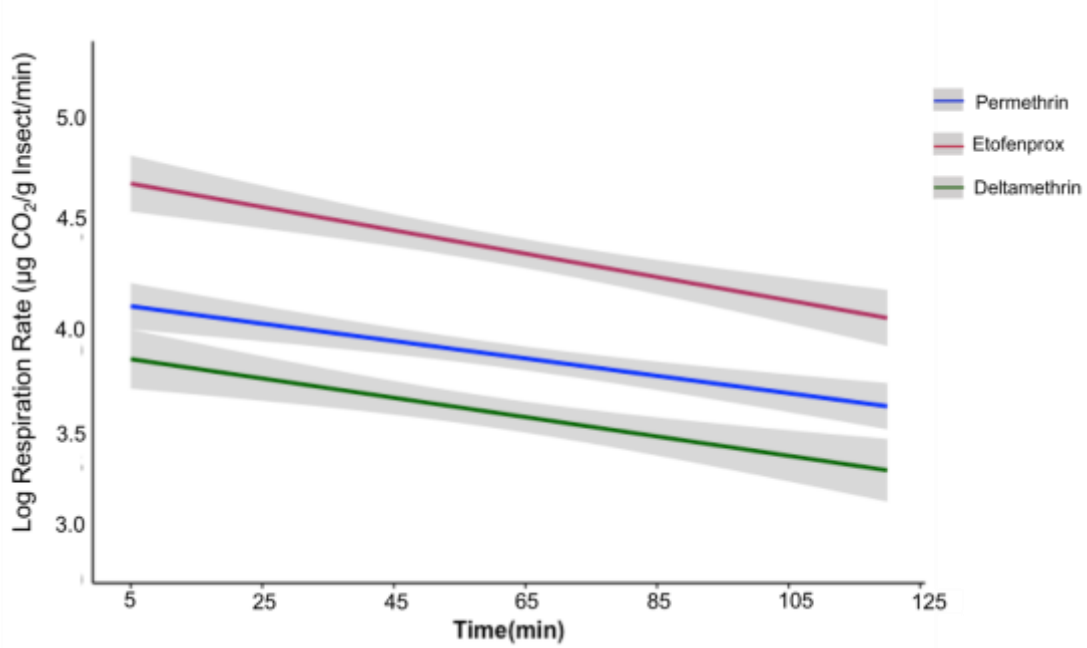

Figure 4. Log changes in respiration as a function of time after dosing at the LD_{50} value where permethrin (blue), etofenprox (red), and deltamethrin (green) are compared to one another. Confidence intervals are represented in the gray-shaded region of each line.

CHAPTER THREE

LEAF RESIDUE TOXICITY AND RISK OF MOSQUITO INSECTICIDES TO THE BEES, *MEGACHILE ROTUNDATA* AND *APIS MELLIFERA*

Contribution of Authors and Co-Authors

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Contributions: Conceived and designed experiments, collected and processed experimental data, analyzed and interpreted results, and wrote manuscript for journal submission.

Co-Author: Michelle L. Flenniken

Contributions: Collaborated with interpretation of results and manuscript preparation and provided *A. mellifera* test insects.

Co-Author: Kevin M. O'Neill

Contributions: Collaborated with interpretation of results and manuscript preparation.

Co-Author: Robert K. D. Peterson

Contributions: Conceived and designed experiments, provided critical input at all stages of experiment including interpretation of results and manuscript preparation.

Manuscript Information Page

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Status of Manuscript:
__X__ Prepared for submission to a peer-reviewed journal
____ Officially submitted to a peer-review journal
____ Accepted by a peer-reviewed journal
____ Published in a peer-reviewed journal

CHAPTER THREE

LEAF RESIDUE TOXICITY AND RISK OF MOSQUITO INSECTICIDES TO THE BEES, *MEGACHILE ROTUNDATA* AND *APIS MELLIFERA*

The following chapter has been prepared for submission to a peer-reviewed journal

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Abstract

The alfalfa leafcutting bee (*Megachile rotundata* F.) (Hymenoptera: Megachilidae) is one of the most intensively managed solitary bees and is an important pollinator of many crops, especially alfalfa (*Medicago sativa* L.) (Fabaceae) in Western North America. However, little is known about its response to insecticides, specifically pyrethroids, which are frequently used to manage populations of adult mosquitoes. Therefore, we conducted a reasonable worst-case exposure scenario where we sprayed pyrethroids at maximum label rates over a flowering alfalfa field, mimicking a mosquito adulticide application using a ground-based ultra-low-volume (ULV) sprayer. We exposed female *M. rotundata* and *Apis mellifera* L. (Hymenoptera: Apidae) to the foliage for 48 h after the application. Insecticides used included two water-based formulations (Aqua-Reslin® (permethrin), and DeltaGard® (deltamethrin)), and an oil-based

formulation (Zenivex®E20 (etofenprox)). There was no significant difference in mortality between the control and treated groups for each of the formulations in both bee species. Results suggest that mortality risk for these species can be managed effectively even if these insecticides are applied over alfalfa.

Introduction

West Nile virus and several other pathogens vectored by mosquitoes have been a major concern as outbreaks continue to occur in the Western Hemisphere (Peterson et al. 2006). These outbreaks have caused insecticides such as pyrethroids to be more widely used to reduce mosquito-borne diseases. Pyrethroids are often used to manage adult mosquito populations because they have a rapid toxic effect on mosquitoes, a short half-life in the environment, and a low toxicity to terrestrial vertebrates (Schleier III and Peterson 2011, Elliot et al. 1973). Although several studies have focused on the toxicity of honey bees, *Apis mellifera* L., to pyrethroids (Felton et al. 1986, USEPA 2015, Hester et al. 2001), little information exists on how other bee species respond to these mosquito adulticide applications. With increased use of these insecticides throughout the U.S., public concern has also increased in regards to the potential impact these insecticides have on non-target organisms (Jensen et al. 1999, Their 2001, Peterson et al. 2006).

These insecticides are commonly applied using a ground-applied an ultra-low-volume (ULV) treatment, which creates an aerosol cloud that targets airborne adult mosquitoes, and are often used to manage high densities of adult mosquitoes (Schleier III and Peterson 2010, Davis et al. 2007). The aerosol cloud released from the applicator is

comprised of extremely small liquid droplets that range from 8 to 30 µm in diameter and are capable of staying aloft in the air.

Several studies have shown that the combination of ULV application with organophosphorus and pyrethroid insecticides have detrimental effects on non-target organisms such as honey bees, beneficial insects, and small flying insects (Rinkevich et al. 2017, Peterson et al. 2016, Jensen 1999, Hill et al. 1971, Hester et al. 2001, Caron et al. 1979). However, the movement of this aerosol cloud allows for a very small percentage of the insecticide to be deposited on surfaces within the surrounding environment, resulting in low toxicity after application (Lofgren et al. 1973, Zhong et al. 2003). Therefore, the purpose of our study is to mimic a worst-case scenario of mortality risk when bees are exposed to leaf surfaces that have been in direct contact with the maximum labeled rate of insecticide.

Despite previous studies that observed the effect of insecticides on *A. mellifera* via aerial and ground-based ULV (ultra-low-volume) application (Caron 1979, Hester 2001, Rinkevich et al. 2017), very few studies have tested how other pollinator species respond to these methods. Therefore, we observed the effects of three different pyrethroid insecticide formulations representing the three types of pyrethroids (Type I, Type II, non-ester) on both the *M. rotundata* and the *A. mellifera*, both of which are important pollinators in the U.S. (Artz and Pitts-Singer 2015, Pitts-Singer and Cane 2011). Understanding how other species respond to these insecticides is crucial to future insecticide management practices and reducing the risk of these insecticides to beneficial organisms.

Materials and Methods

Insects

Megachile rotundata. Diapausing *M. rotundata* larvae in loose leaf cells were purchased from JWM Leafcutters, Inc. (Nampa, Idaho) in April 2015 and 2016 and were placed in room temperature (23°C) for three days before being placed in the rearing room set to 28 ± 2°C, relative humidity (RH) 40-60%, and a photoperiod of 16:8 (L:D) hours; post-diapause rearing of *M. rotundata* at 28°C results in high emergence rates and adults with high lipid content (O'Neill et al. 2011). Cells of *M. rotundata* were placed inside Specimen Transfer Cages No-See-Um Mesh, 61 x 61 cm (BioQuip Products, Inc., Rancho Dominguez, California) and reared for 15-28 days. As adults began to emerge from leaf cells, individuals were removed from the cage using an aspirator and then transferred for approximately 20 min to a refrigerator set at 4°C. Once the bees were immobile, they were removed from the container and sorted by sex on a Laboratory Chill Table (BioQuip Products, Inc., Rancho Dominguez, California) and only females were collected to perform the assay. After females were sorted from males, 25 females were placed in 20 mL glass scintillation vials (Thermo Fisher Scientific Co., Waltham, Massachusetts) and stored for no longer than 24 h before dosing.

Apis mellifera. Frames of newly emerged honey bees were obtained from colonies maintained at Montana State University (MSU) in Bozeman, Montana. Teneral female worker (nurse) bees (approximately 24 h post-emergence) were used for this study to ensure same-age individuals as per USEPA (2012) standard experimental procedures.

The brood comb of bees was stored in a warming chamber set to 32 ºC which was placed inside of a 49.2 L Coleman Xtreme Cooler™ (Kingfisher, Oklahoma) and installed with a STC-1000 temperature controller (AGPtek®, Brooklyn, New York). A total of 75 female worker bees were used for each treatment with 125 used for each replicate. To ensure the honey bee specimens were the most robust before treatment, bees were kept in the warming chamber until the start of the experiment.

Ultra-low-volume Applicator and Formulations

A GUARDIAN® 95 ES (Adapco, Sanford, Florida) ULV truck-mounted aerosol generator was used to make all insecticide applications. Each insecticide formulation was calibrated such that the maximum-labeled rate of insecticide (0.017 kg/ha) would be applied with a 1:1 dilution of either deionized water or mineral oil (STE Oil Company, Inc., San Marcos, Texas). The aerosol generator was mounted on a pickup truck with the spray head angled at 45° above horizontal (Fig. 5).

Permethrin ((3-phenoxyphenyl)methyl 3-(2,2-dichloroethenyl)-2,2dimethylcyclopropane carboxylate), was used as a water-based formulation (Aqua-Reslin®); etofenprox (2-(4-ethoxyphenyl)-2-methylpropyl 3-phenoxybenzyl ether), was used as an oil-based formulation (Zenivex®E20), and deltamethrin (DeltaGard®) ((S)-α-cyano-3-phenoxybenzyl (1R,3R)-3-(2,2- dibromovinyl)-2,2-dimethylcyclopropane carboxylate) was used as a water-based formulation. The calibration of Aqua-Reslin® was 192.23 mL/min (flow rate 6.5 oz/min), Zenivex®E20 was calibrated to 212.93 mL/min (7.2 oz/min) and DeltaGard® was 359.90 mL/min (12.2 oz/min).

Experimental Design

The field site was located at the Montana State University Horticulture Farm in Bozeman, Montana (45°39’32.2” N, 111°04’21.3” W) for both summer 2015 and summer 2016. The plot was a square 71 x 71 m grid divided into 4 quadrats and separated by a cross-section drive line of 10-m wide (Fig. 6). Rotating slide impingers (Leading Edge Associates, Inc., Fletcher, North Carolina) were randomly placed in two of the four quadrats to ensure the insecticide entered the treated subplots and did not enter the control subplots. Impingers rotated both Teflon- and magnesium oxide-coated slides (for both oil- and water-based formulations, respectively). After each insecticide application, slides were immediately brought back to the laboratory and inspected for the presence of droplets and were analyzed using DropVision® measuring system (Leading Edge Associates, Inc., Fletcher, NC, USA) (Peterson et al. 2016) (Table 2). The design of this plot was created such that it was possible to shift control and treated plots with wind direction. To ensure that there was no residual effect from prior applications, a period of no less than 48 h was imposed between each field application.

To mimic the applied practice of using a ULV truck-based treatment for adult mosquito management while still being reasonable worst case, insecticide was applied such that the aerosol cloud moved above and over the flowering alfalfa canopy (Fig. 1). Sprays occurred during the evening hours of 7:30 and 9:00 p.m. when the wind was between 5 and 10 mph (mean wind speed 6.1 mph ± 3.0 mph) and temperature was 28.6ºC ± 3.6ºC. Foliage was collected the following day, approximately 12 h later. Following USEPA (2012) protocol, only the top 15 cm of the alfalfa was collected to

ensure only the exposed foliage was used during the trials. A diagonal transect line was walked for each quadrat and a random number generator was used to determine how far from the transect line the foliage was collected. Collection of foliage occurred no less than 8 times for each quadrat and foliage was mixed by treatment to be sure there was no bias on foliage distance from each application. Once enough foliage was collected, the alfalfa samples were brought to the laboratory, mixed (by treatment) and cut into 5-cm sections (USEPA 2012), each of which contained multiple leaves.

Once the foliage was prepared, 500 mL of foliage was placed into Sterlite® 35.9 cm x 20 cm x 12.4 cm plastic containers (Sterlite Corporation, Townsend, Massachusetts) along with cotton swabs soaked in a 1:1 by weight dilution of pure cane sugar (Domino Foods, Inc., Brooklyn, New York) and deionized water. Twenty-five *M. rotundata* or *A. mellifera* females were then placed in the containers in a room set to 30ºC ± 2.2ºC for 48 h with 40-60% RH, and a photoperiod of 16:8 (L:D) hours. For each replicate, each treatment (control and active ingredient) had three subsamples with 25 bees in each box (n = 75 per treatment, n = 125 per replication) except for two *A. mellifera* replications because not enough nurse bees emerged from the brood comb the previous day. For one Zenivex® trial, only 92 nurse bees emerged, therefore, n = 46 for the treated group and n = 46 for the control group; and for one DeltaGard® trial, only 132 nurse bees emerged, and the population was evenly split like the Zenivex® trial (n = 66 for each group). Mortality was recorded at the end of each 48-hr period. A bee was considered dead when completely immobile and unresponsive to probing.

Statistical Analysis

For each bee species, experiments for each active ingredient were replicated a total of four times with three subsamples of populations for both the control and treated groups (i.e., 175 bees per replication, except two of the honey bee trials where the total number of bees was 96 and 132, respectively). Replicates were not used in the analysis if mortality was greater than 20% in the control groups (Abbott 1925, Yu 2008). Percentage mortality was averaged over the subsamples for each replication and then a Welch's Two-Sample t-Test was used to determine significant differences in mortality between the populations (R Studio, Inc. 1.0.136). The data were arcsine transformed and a Welch's t-test was used to account for the heterogeneity of the variance within the populations.

Results and Discussion

The mean percentage mortality for adult *M. rotundata* and *A. mellifera* was compared between treated and control groups within each species. There were no statistically significant differences in the means of control and treated groups for both *M. rotundata* and A. *mellifera*.

For *M. rotundata*, the mean percent mortality for Aqua-Reslin® was 16.9% (SE = 0.8) control group and 24.2% (SE = 7.1) for the treated group (P = 0.39). Percent mortality for Zenivex® was 15.4% (SE = 1.8) for the control group and 22.8% (SE = 6.0) for the treated group (P = 0.31). The control group for DeltaGard® had a mean of 19.0% (SE = 1.9) whereas the treated group had a mean of 20.9% (SE = 3.7) (P = 0.67) (Fig. 7).

Similar to the response of *M. rotundata* to the three pyrethroids, there was no significant difference in mortality between the control and treated groups of *A. mellifera*. For Aqua-Reslin®, the mean was 10.67% (SE = 1.5) for the control group and 48.43% (SE = 11.6) for the treated group (P = 0.10). Percent mortality for Zenivex® was 11.6% (SE = 5.9) for the controls and 55.2% (SE = 14.6) for the treated (P = 0.14). For DeltaGard® means were 15.8% (SE = 2.1) for controls and 46.6% (SE = 5.3) (P = 0.061) for the treated groups, respectively (Fig. 8).

For *A. mellifera*, the data were arcsine transformed and Welch's Two-Sample t-test was used because of the variability in mortality within both groups. We suspect that because these were teneral nurse bees, they were unable to adjust to the conditions in the test boxes and this ultimately resulted in their inconsistent response. We did not, however, observe this behavior among *M. rotundata*, perhaps because *M. rotundata* test bees were at most 24 h older than the honey bees and they are not a eusocial species. The high sensitivity of *A. mellifera* could be because of the lower number of genes encoding xenobiotic detoxifying enzymes within its genome. This deficit of detoxification genes may not be exclusive to *A. mellifera*, but may be a specific adaptation within the eusocial insect group as other solitary Apiformes typically have more of these enzymes within their genome (Claudianos et al. 2006, Hardstone and Scott 2010, Arena and Sgolastra 2014). Future studies should focus on evaluating foraging-age honey bees as they are more likely to be exposed to insecticide during their foraging flights compared with in-hive nurse bees (Alburaki et al. 2017). However, a disadvantage with using foraging-age

bees is variability in ages and therefore possibly greater variability in responses, though could perhaps be estimated using examination of wing wear patterns.

Although pyrethroids are highly toxic to bees, other studies have shown that when non-target organisms are not in direct contact with the very small droplets of the mosquito insecticide, sheltered either by obstacles or being a sufficient distance away from the spray source, there is little mortality (Rinkevich et al. 2017, Peterson et al. 2016). Caron (1979) applied malathion via a ground-based ULV application to caged *A. mellifera* of varying ages during the daytime and saw significant mortality but the insecticide had no measurable effect on the colonies during the nighttime application. Similarly, Hester et al. (2001) showed that when malathion was applied undiluted at the maximum labeled rate in open and forested areas, there was minimal impact of this insecticide on *A. mellifera* colonies. Alburaki et al. (2017) observed no significant mortality in 20-22-day old foraging *Apis mellifera carnica* when exposed to high levels of two pyrethroids, cyhalothrin and bifenthrin in agricultural crop pollen. These studies in addition to our results indicate that *M. rotundata* mortality may be mitigated by targeting the time of insecticide application to when bees are less active (i.e., early morning or late evening hours) or possibly by covering the shelters at night such that nests are not exposed to the insecticide (Hester et al. 2001, Zhong et al. 2003).

Pyrethroid deposition on leaf surfaces also plays an important role in non-target organism risk to insecticides. Pyrethrins are highly photolabile and are known to have a half-life <5 h in direct sunlight, which greatly reduced their ability to be used in commercial agriculture (Schleier III and Peterson 2011, Crosby 1995). This resulted in

the formulation of pyrethroids, which still have a low half-life, but have a longer residual for effectiveness against pests (Antonious 2004, Schleier III and Peterson 2011).

However, when applied using ULV technology for mosquito management, only 1 to 30% of pyrethroid insecticides deposit on the ground within 100 m of the spray source (Knepper et al. 1996, Schleier III and Peterson 2010, Tietze et al. 1994, Schleier III et al. 2012). The combination of low deposition percentages and photolability of pyrethroids used for adult mosquitoes, in outdoor space treatments suggests that *A. mellifera* and *M. rotundata* are at low risk and may not experience appreciable mortality as a result of these outdoor mosquito control measures. However, *A. mellifera* and *M. rotundata* morbidity in the context of these insecticide treatment regimens remains an outstanding question.

The results presented in this study provide insight into management practices for mitigating the risk of adult mosquito insecticides to non-target organisms, especially when applying the chemicals during hours when they are least active. Some, but not all, insecticide labels provide specific instructions on where and when to apply the formulation. For example, the Zenivex® E20 label specifies that applications should not occur on blooming crops or weeds when bees are visiting the area or, if unavoidable, the timing of the application should provide the maximum possible interval between treatment and the next period of bee activity. In accordance with these labels and by applying these insecticides via an ULV system and avoiding application during peak flying times of *M. rotundata* and *A. mellifera*, the risk of mortality can be minimized due to the small deposition and rapid degradation rate of pyrethroids.

Acknowledgements

We thank members of the MSU CoBRA Lab: G. Sinrud and J. Schmidt for their help analyzing droplet data and experimental setup, C. Donahoo for her assistance in data collection and experimental setup, C. Brown for his help with experimental setup, and C. Preftakes for assistance with statistical analysis, aerosol generator troubleshooting, and experimental setup. We also thank JWM Leafcutters, Inc. and the Flenniken Lab for providing the test bees. A special thanks to R. Brekke and J. Heen for allowing us to use the Montana State University Bozeman Agricultural Research and Teaching Farm as a research site for both field seasons; and to J. Stroud (Adapco) and D. Gaiser (Central Life Sciences) for calibration of the aerosol generator and donation of pyrethroid insecticides. The research was funded by the Mosquito Research Foundation, the Montana Agricultural Experiment Station, and Montana State University. This material is based on work that is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, Hatch Multistate Project No. W-3045.

Figure 5. Ultra-low-volume applicator releasing a formulation over an alfalfa field.

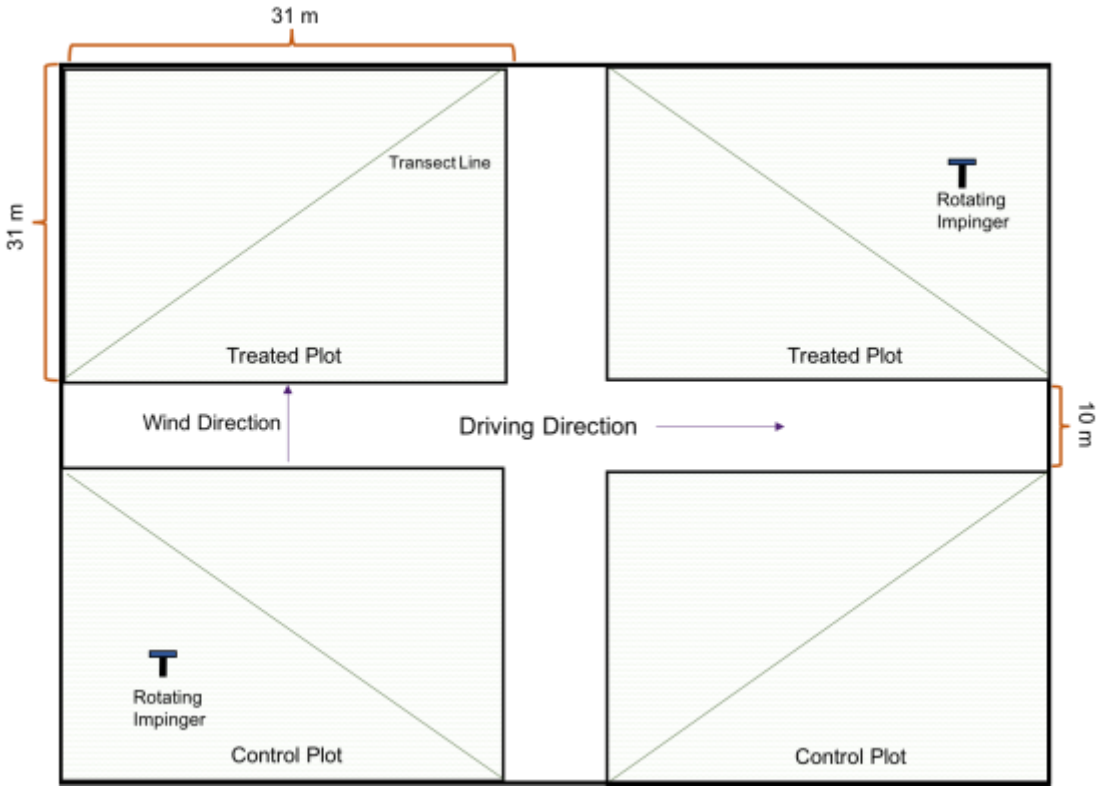

Figure 6. Diagram of an experimental plot used to collect alfalfa foliage. Each quadrat is 31 m x 31 m and intersected by a 10-m drive line. Rotating slide impingers were randomly placed in two plots to ensure the insecticide either did or did not enter the treated or control plot, respectively.

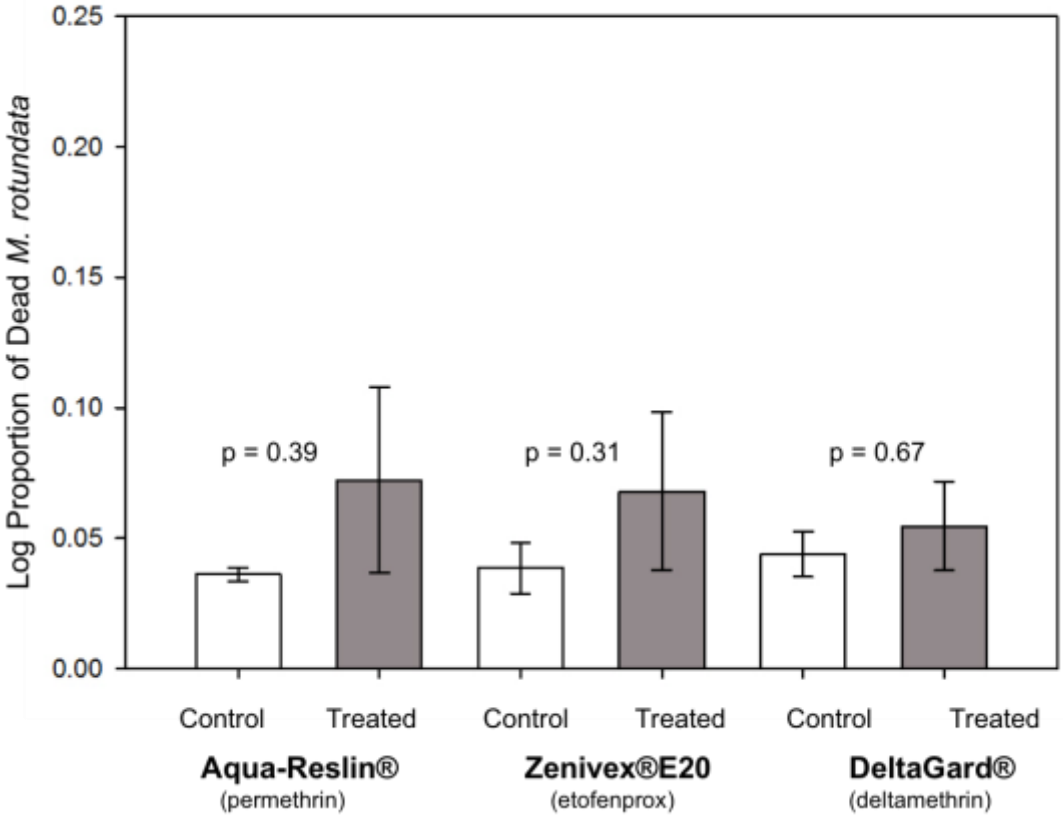

Figure 7. The response of adult female *Megachile rotundata* to three pyrethroids. Gray bars represent percent mortality for treated groups and white bars represent percent mortality for control groups. The mean mortality was 24.17%, 22.82%, and 20.83% for Aqua-Reslin®, Zenivex®, and DeltaGard® treatments, respectively. Mean mortality for the control groups was 16.93%, 15.39%, and 18.96%.

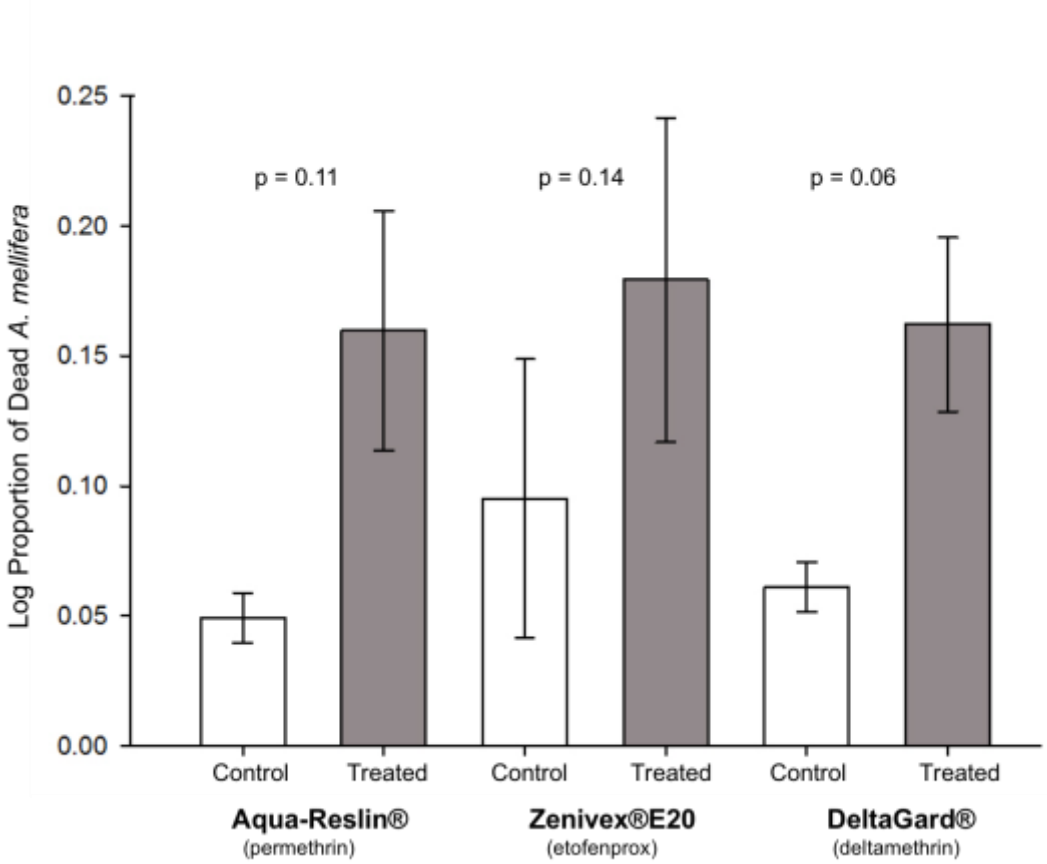

Figure 8. The response of adult female *Apis mellifera* to three pyrethroids.
Gray bars represent percent mortality for treated groups and white bars represent percent mortality for control groups. The mean mortality was 48.48%, 55.2%, and 46.55% for Aqua-Reslin® (permethrin), Zenivex® (etofenprox), and DeltaGard® (deltamethrin) treatments, respectively. Mean mortality for the control groups was 10.67%, 11.56%, and 15.17%.

Table 2. Droplet density and abundance for each insecticide formulation.
Volume median diameter (VMD) of the droplets; the number of droplets collected; and the relative span factor (RSF), indicating the uniformity of the drop size distribution, are recorded for each pair of slides.

Treatment	**VMD (µm) (SE)**	**Droplets collected (SE)**	**RSF (SE)**
Aqua-Reslin®	8.41 (0.79)	229.75 (61.92)	0.83 (0.04)
Zenivex®	9.94 (0.55)	272.51 (33.40)	0.86 (0.05)
DeltaGard®	9.71 (0.88)	235.01 (27.68)	0.74 (0.06)

CHAPTER FOUR

THE EFFECTS OF AN ULTRA-LOW-VOLUME APPLICATION OF ETOFENPROX FOR MOSQUITO MANAGEMENT ON *MEGACHILE ROTUNDATA* (HYMENOPTERA: MEGACHILIDAE) LARVAE AND ADULTS IN AN AGRICULTURAL SETTING

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Contributions: Collaborated with interpretation of results and manuscript preparation.

Co-Author: Kevin M. O'Neill

Contributions: Collaborated with interpretation of results and manuscript preparation.

Co-Author: Ruth P. O'Neill

Contribution: Constructed nest shelters and provided critical information on experimental design and data collection.

Co-Author: Casey M. Delphia

Contribution: Provided critical information on experimental design and data collection.

Co-Author: Robert K. D. Peterson

Contributions: Conceived and designed experiments, provided critical input at all stages of experiment including interpretation of results and manuscript preparation.

Manuscript Information Page

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Status of Manuscript:
__X__ Prepared for submission to a peer-reviewed journal
____ Officially submitted to a peer-review journal
____ Accepted by a peer-reviewed journal
____ Published in a peer-reviewed journal

CHAPTER FOUR

THE EFFECTS OF AN ULTRA-LOW-VOLUME APPLICATION OF ETOFENPROX FOR MOSQUITO MANAGEMENT ON *MEGACHILE ROTUNDATA* (HYMENOPTERA: MEGACHILIDAE) LARVAE AND ADULTS IN AN AGRICULTURAL SETTING

The following chapter has been prepared for submission to a peer-reviewed journal

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Abstract

The alfalfa leafcutting bee, *Megachile rotundata* F. (Hymenoptera: Megachilidae) is one of the most intensively managed solitary bees and greatly contributes to alfalfa production in both the United States and Canada. Although production of certain commodities, especially alfalfa seed, has become increasingly dependent on this species' pollination proficiency, little information is known about how *M. rotundata* is affected by insecticide exposure. To better understand the risk posed to *M. rotundata* by the increasing use of insecticides to manage mosquitoes, we conducted field experiments that directly exposed *M. rotundata* nests, adults, and larvae to a pyrethroid insecticide via a

ground-based ultra-low-volume (ULV) aerosol generator. We directly targeted nest shelters with Zenivex® E20 (etofenprox) at the half-maximum rate of 0.0032 kg/ha at dusk and then observed larval mortality, adult mortality, and the total number of completed nests for both the treated and control groups. There was no significant difference in the proportion of dead (P = 0.99) and alive (P = 0.23) larvae when the control group was compared to the treated group. We also did not observe a significant difference in the number of emerged adults reared from the treated shelters (P = 0.22 and 0.50 for females and males, respectively), and the number of completed cells after exposure from the insecticides continued to increase throughout the summer, indicating that provisioning adults were not affected by the insecticide treatment. The results from this study suggest that the amount of insecticide reaching nest shelters may not be sufficient to cause significant mortality.

Introduction

The alfalfa leafcutting bee, *Megachile rotundata* F. (Hymenoptera: Megachilidae), has nearly tripled alfalfa (*Medicago sativa* L., Fabaceae) seed production since its introduction to the U.S. in the 1940s (Pitts-Singer and Cane 2011). This solitary bee has surpassed the efficiency of honey bees in the same crop, *Apis mellifera* L. (Hymenoptera: Apidae), primarily due to the ability of *M. rotundata* to trip the staminal columns of *M. sativa* as much as 80% of the time when visited, far exceeding the ability of the honey bee (22% of visited flowers tripped) for this particular plant (Pitts-Singer and Cane 2011, Cane 2002). The capability of *M. rotundata* to pollinate alfalfa (as well

as other crops such as canola, cranberries, and blueberries) so efficiently, as well as its coincided emergence with peak alfalfa bloom, has resulted in this species becoming the most intensively managed solitary bee, a pollinator second only to *A. mellifera* (Pitts-Singer and Cane 2011).

Of the $14 billion value attributed to *A. mellifera* pollinated U.S. crops, *M. rotundata* pollination is responsible for one-third of that total value. *M rotundata* pollinates two-thirds of the world's alfalfa crop, which is primarily used to feed livestock, especially dairy cattle (Pitts-Singer 2008, Van Deynze et al. 2008, Pitts-Singer and Cane, 2011).

Although *M. rotundata* is the most intensively managed solitary bee, research has primarily focused on the effect of insecticides on *A. mellifera*, and rarely focused on the effects on other bee species (Caron 1979, Hester 2001, Rinkevich et al 2017). Solitary bees are important pollinators of plants in wild and agricultural settings. Although they play a crucial role in both residential and commercial settings, solitary bees have been largely ignored in pesticide regulations (Blacquière *et al.* 2012, Sandrock et al. 2013). We therefore aimed to assess how pyrethroids, a commonly used insecticide class for the management of mosquitoes and other pest insects, affects both an existing and a future generation of *M. rotundata*.

Pyrethroids represent approximately 23% of the global insecticide market and with over 3,500 registered formulations worldwide, they are one of the most commonly used classes of insecticides today (Schleier II and Peterson 2012, USEPA 2015). For this study, we examined the effects of a formulated version of etofenprox, a non-ester

pyrethroid. This type of pyrethroid prolongs the opening of voltage-gated sodium channels (VGSC) by inducing repetitive discharges (Nishimura et al. 1996), which is similar to type I pyrethroids where the VGSC is modified such that there is a slight prolongation that causes multiple long action potentials.

A common application of these insecticides for mosquito management in both agricultural and residential settings is by ultra-low-volume (ULV) aerosol generators. Sprayed formulations via ULV application tend to stay aloft in the air and therefore result in very low deposition rates of the insecticide in the surrounding environment (Lofgren et al. 1973). The average droplet size is extremely small and ranges from 8 to 30 µm diameter and the droplets are capable of staying aloft in the air for prolonged periods and so be more likely to contact airborne mosquitoes. This method, in combination with pyrethroid insecticides, which have a short half-life within the environment, are highly photolabile, and have a rapid knockdown effect of targeted flight mosquitoes (Schleier and Peterson 2011, Crosby 1995) results in an efficient way to manage mosquito populations. However, the risks of these methods on solitary, non-target, bee species are still largely unknown.

Several studies have shown that the combination of ULV application with organophosphorus and pyrethroid insecticides have detrimental effects on non-target organisms such as honey bees, beneficial insects, and small flying insects (Rinkevich et al. 2107, Peterson et al. 2016, Jensen 1999, Hill et al. 1971, Hester et al. 2001, Caron et al. 1979). However, the effect of the insecticide aerosol on *M. rotundata* nests and developing offspring is undocumented. Unlike the eusocial honey bee, *M. rotundata* is a

solitary species in which each female separately cares for her own brood in her own individual nest cavities. Although *M. rotundata* is a solitary species, it is also highly gregarious and commercial nest shelters for this species consists of thousands of small cavities in which females provision nests. The different life history compared to eusocial bees may result in dissimilar responses when exposed to insecticides. We therefore examined the effect of Zenivex® E20 (etofenprox) on foraging and provisioning adults and developing larvae of *M. rotundata* to observe how this insecticide affects the population when nests come in direct contact with the aerosol produced by the ULV applicator.

Materials and Methods

Insects

Diapausing *M. rotundata* larvae in loose leaf cells were purchased from JWM Leafcutters, Inc. (Nampa, Idaho) in April 2016 and kept in cold storage until early summer to coincide with alfalfa bloom. In mid-June, bees were kept at room temperature (23°C) for three days before being placed in the rearing room set to 28 ± 2°C, relative humidity 42-60%, and a photoperiod of 16:8 (L:D) hours; post-diapause rearing of *M. rotundata* at 28°C results in high emergence rates and adults with high lipid content (O'Neill et al. 2011). Cells of *M. rotundata* were placed inside Specimen Transfer Cages No-See-Um Mesh, 61 x 61 cm (BioQuip Products, Inc., Rancho Dominguez, California) and reared for 10 days, at which time, all were still pupae. The typical developmental time for *M. rotundata* from prepupal to adult is 25-30 days at approximately 28°C (O'Neill et al. 2011). We chose to develop the bees for 10 days to efficiently transport the

loose cells from the rearing room to the field site with minimal stress. The procedure followed is roughly based on the four basic stages of management of *M. rotundata* bee management in the loose cell system. The four stages of this practice are: late-spring incubation, summer release and nesting, early fall removal of loose prepupal cells, and wintering of prepupal cells (Pitts-Singer 2008). We followed this protocol because it is the most common practice in production systems for *M. rotundata* today.

Experimental Design

Experiments were conducted in two alfalfa fields in Bozeman, Montana in the summer of 2016. The first location (Plot 1) was at 45º42’50.1”N, 111º09’26.6”W and the second location (Plot 2) was at 45º41’54.5”N, 111º07’44.9”W. At location were placed four *M. rotundata* nest shelters containing wood laminates that that provided nest tunnels for the bees (Fig. 9). Two shelters at each location were treated with insecticide and two served as controls.

Each nest shelter had a total of 1,430 nest cavities available for nest construction. Female *M. rotundata* are capable of laying two eggs per day and may complete under ideal conditions as many as cells over their 7- to 8-week life span when floral resources are abundant (Pitts-Singer and Cane 2011, Maeta and Kitamura 2005). Due to the average length of an individual cell, we estimated that one row could fit 12 (actual number 8.3 ± 0.49) individual cells. One female could therefore fill approximately 5 tunnels with eggs over the course of her life time. As a conservative measure to maximize the number of cavities in the nest shelters that would be provisioned with next generation

M. rotundata, we assumed one female would fill 3 rows throughout the summer. Based on the calculations provided by JWM Leafcutters, Inc., one gallon of loose cells was approximately 10,000 bees. The sex ratio of *M. rotundata* has been reported as 1:3 (female:male) (Gerber and Klostermeyer 1972, Gerber and Klostermeyer 1970,), or 1:2 (females:males) (Richards 1993). However, reports from JWM Leafcutters, Inc. estimates a 1:3 (females:males) ratio and we therefore estimated that there were 3,300 females in one gallon of loose cells, if all cells are healthy. Assuming the number of live bees per gallon, we then estimated 1.5 L of loose cells would be required to fill 1,430 nest rows off offspring. The 1.5 L of loose cells were placed within each shelter approximately 5 days before anticipated peak male emergence. Cells were placed in Sterlite® 35.9 cm x 20 cm x 12.4 cm plastic containers without lids and set out in each nest shelter. The purpose of the plastic containers was not only for transport of the loose cells, but as protection against severe weather and predators.

After 3 weeks of setting bees in the nest shelters, female *M. rotundata* had provisioned and filled approximately one-half of the nests in each bee board. We chose this time to apply a one-time application of Zenivex® E20 (etofenprox) directly to the shelters via a ground-based ULV applicator at the half-maximum label rate of 0.0032 kg/ha for mosquito control and diluted at a 1:1 ratio of mineral oil (STE Oil Company, Inc., San Marcos, Texas) from a distance of 23 m from the opening of the nest shelter; 23 m is 25% of the 91 m effective spray swath. For this procedure, a GUARDIAN® 95 ES (Adapco, Sanford, Florida) ULV truck-mounted aerosol generator was used to make all insecticide applications. Etofenprox (2-(4-ethoxyphenyl)-2-methylpropyl 3-

phenoxybenzyl ether), was used as an oil-based formulation (Zenivex® E20). Zenivex® E20 was calibrated to 109.4 mL/min (3.6 oz/min).

Four shelters were placed at each location, 90 m apart from each other along a strip of alfalfa 488 m long and 45.7 m wide. Shelters were positioned to face east so that the faces of nest boards received maximum sunlight in the morning, but shade in the afternoon. Before each insecticide application, all completed (capped) nests were marked with a red grease pencil. The number of completed nest cavities was counted again when shelters were removed from the field at the end of the growing season and compared to the number just before each spray.

For each location, we randomly assigned two shelters each were randomly selected as either a control or treated shelter (four shelters total). All bees were reared and maintained within the field under the same conditions, except for application of the insecticide. The one-time application of Zenivex® E20 per each location was conducted on July 26, 2016 and July 27, 2017 at 8:57 pm and 8:14 pm MST with wind speeds of 7.1-9.4 mph and 5.5-9.1 mph for Plot 1 and Plot 2, respectively. Immediately before each application, 2.4 m x 3.6 m two-layer drop cloth (Ace Hardware Corp., Oak Brook, Illinois) was placed over the front of each control shelter to ensure the insecticide application did not drift on the control shelters. The cloth coverings were removed from the control shelters within 5 min after the application. In combination with the cloth coverings, a buffer zone (area where aerosol generator was turned off) was implemented in which the drive line was 1.2 times as long as the sample line (Fig. 10) (ASAE 2013). The aerosol generator applied the insecticide approximately 23 m from the front of the

targeted shelter. Rotating slide impingers (Leading Edge Associates, Inc., Fletcher, North Carolina) were placed 1.5 m from each nest shelter to verify that the insecticide entered the treated areas and did not enter the control areas. Impingers rotated 75 x 25 mm Teflon-coated slides at a speed of 5.6 m/s and were positioned 18.4 cm apart from center to center. After each insecticide application, slides were immediately brought back to the laboratory and inspected for the presence, number, and size of droplets using DropVision® measuring system (Leading Edge Associates, Inc., Fletcher, NC, USA) (Peterson et al. 2016) (Table 3). Insecticide deposited on slides near all treated nest shelters, but not on any of the slides near the control shelters. Moreover, for each application we observed the insecticide aerosol cloud move over the alfalfa canopy and through each treated nest shelter.

Immediately following the spray, 2.4 m x 3.6 m two-layer drop cloth (Ace Hardware Corp., Oak Brook, IL) were placed directly in front of each shelter and staked to the ground to catch adults dying after application. The next morning, approximately 12 h later, if there were no dead bees in front of the shelter, the drop cloth was removed from the field. The purpose of the drop cloth was to potentially capture any dead adults the following 12 h after the insecticide application.

On August 26, 2016 nest boards were removed from the field and brought to the laboratory where they were kept at room temperature (23°C). Dissection of individual leaf cells started on August 28, 2016 and continued until all 400 nest cavities (50 per box) were completed on October 16, 2016. For the dissection of individual cells, a random number generator was used to select 50 nests from each nest shelter. For control shelters,

a mean of 432 ± 18.0 cells (1,729 total) was dissected; a mean of 452 ± 97.5 cells (2,260 total) from the treated shelters was dissected. We randomly selected 50 nests throughout each shelter to characterize the number of live and dead larvae in each box. The cells in each cavity were then dissected and were categorized as 1) having dead larvae killed by parasitoid wasps, 2) having dead larvae, but with cause of death "unknown", 3) have a unconsumed "pollen ball" (i.e., the presence of pollen provisions, but with no evidence of larval feeding); and "total dead", which is the sum of all dead larvae.

The remaining larvae were then stored for the winter, similar to the protocol used in the loose-cell storage system, and stored at 7°C for six months (O'Neill et al. 2011, Bohart 1962). After six months, leaf cells were placed in room temperature (23°C) for three days before being placed in the rearing room set to 28 ± 2°C, relative humidity (RH) 40-60%, and a photoperiod of 16:8 (L:D) hours; post-diapause rearing of *M. rotundata* at 28°C results in high emergence rates and adults with high lipid content (O'Neill et al. 2011). Cells were housed in eight Specimen Transfer Cages No-See-Um Mesh, 61 x 61 cm (BioQuip Products, Inc., Rancho Dominguez, California), one cage for each shelter and reared for 15-28 days. Because some nest boxes were filled with more cells than others, a standard of 532 mL of cells was placed into each rearing cage (mean = 88.5 g, SE ± 1.9) from each nest shelter. Each day, newly emerged *M. rotundata* adults were removed from each cage using an aspirator, sexed, and counted so the total number emerging from each shelter was recorded.

Statistical Analysis

The proportion of larvae was recorded for each of the five categories (number alive, total dead, death due to unknown cause, death by parasitism, or presence of pollen ball without evidence of larval feeding) for each of the eight nest shelters across two plots. After determining there was no replication effect between the two field sites by performing an ANOVA on both sites and finding no statistical difference, an ANOVA test was used to compare each of the four categories against one another and then a pair-wise t-test was used to assess the significant difference in mean proportion of individuals for each treatment and for each of the four cell condition categories (R Studio, Inc. 1.0.136). Similarly, the total number of emerging bees in 2017 was compared across treatments using a Welch's t-Test ($\alpha = 0.05$) to account for the unequal variance between each sex and treatment. Data were log-transformed to meet the assumption of normal distribution (R Studio, Inc. 1.0.136). We also compared the number of capped cells before the insecticide application to the number of capped cells at the end of the season by using a t-test to compare means of each treatment. All four boxes across both of the plots were combined for this analysis.

Results and Discussion

The number of droplets and the average size of the droplets for each location provided evidence that the aerosol cloud effectively passed through the treated areas (Table 3). Thus, we can safely assume that the Zenivex®E20 contacted the nest shelters.

However, despite the aerosol contacting the shelters, we found no difference between treated and control shelter in the number of live larvae, and the number of failed cells in each category (Fig. 11).

There was no statistically significant difference at the time of cell dissections in the proportion of larvae when the proportion alive, total dead, unknown dead, parasitoid dead, or pollen ball dead was compared between treatments (Fig. 11). Similarly, the number of emerged adults after winter storage did not significantly differ between treatments when compared by sex. For males, the logged mean of the control group was 5.379 (SE = 0.405) and the mean of the treated group was 5.047 (SE = 0.208) ($P = 0.501$). The logged mean of the female control group was 3.646 (SE = 0.277) and the mean of the treated group was 3.20 (SE = 0.137) ($P = 0.216$). Although there was no significant difference within each sex when compared between treatments, there was a significant difference in the total number of males and females ($P < 0.001$) (Fig. 12). This bias in total number of emerged males and females is not treatment-related and is most likely due to male-biased sex ratio commonly observed in this species (Gerber and Klostermeyer 1972, Gerber and Klostermeyer 1970, O'Neill 2004, Sandrock et al. 2013).

We also compared the number of completed nest cavities before and after each insecticide application to better understand how provisioning adults were affected. The number of completed nest cavities the day before each application was used as a baseline when comparing the number of completed nest cavities on subsequent days (Table 4). These data were recorded to ensure boxes were still being actively filled by provisioning *M. rotundata* adult females after the insecticide application. Overall, there was an

increase in nest construction in the days and weeks after the boxes were exposed to the insecticide. The lack of mortality recorded the following day after each spray and the consistent increase in cells provisions suggests that adult *M. rotundata* did not experience adverse effects after exposure.

Pyrethroids are highly toxic to bees when they are directly exposed (Torchio 1983). However, several studies have shown that when non-target insects are not in direct contact with ULV-applied insecticides, there is often insignificant mortality between the control and treated areas (Rinkevich et al. 2017, Peterson et al. 2016, Caron 1979). In most cases, the level of direct contact is determined by the distance an application occurred from a population as well as physical barriers within a setting. Hester et al. (2001) found that when the organophosphate insecticide malathion was applied using a ground-based ULV applicator for mosquito management, no significant additional adult *A. mellifera* mortality was observed at hives located 7.6 and 15.2 m from the spray source, but nearly 100% of the mosquito population died. In our study, the nest shelters were 23 m from the spray source, which is 25% of the effective spray swath of 91 m.

Similarly, multiple studies have shown that when non-target insects are directly blocked by obstacles from receiving the insecticide aerosol during application, there is little to no mortality (Peterson et al. 2016, Rinkevich et al. 2017, Hester et al. 2001, Caron 1979). Mortality of non-target species that do not come in direct contact with the airborne insecticide is likely mitigated due to the rapid breakdown of pyrethroids within environments as well as minimal deposition of droplets from ULV applicators within the 91 m spray swath. Some studies have found evidence that insecticide residues may

contaminate pollen and leaf foliage if insecticides are repeatedly deposited and consequently result in larval mortality (Zhu et al. 2014). However, we did not observe a significant difference between the control and treated groups throughout the growing season, suggesting that the application did not affect population numbers. Although we did not directly test for the concentration of insecticides in the pollen fed to larvae exposed to Zenivex® E20, we did not observe any significant mortality between larvae in the control group compared with larvae in the treated group and there was no significant difference in the total number of adults emerging after the winter storage period. However, future studies should also measure possible sublethal effects on larvae that carry over into adults of the next season and effect their foraging and reproductive activities.

Overall, our results indicate that there were no detectable deleterious effects on *M. rotundata* when exposed to an application of Zenivex® E20 for adult mosquito management in a field setting during active foraging and nest provisioning. The risks may be further reduced by ensuring that no nest shelters are within spray swaths, which can be relatively easy to manage by turning off aerosol generators as they pass areas of active *M. rotundata* production.

Traditionally, only *A. mellifera* has been intensively studied as a non-target insect for purposes of pesticide regulation. However, this might not be the best choice for a surrogate species due to its complex eusocial life history (Arena and Sgolastra 2014, Hardstone and Scott 2010). *Megachile rotundata* possibly could serve as a model species for other solitary bees, or at least provide evidence that a wider variety of species is

needed to more completely comprehend how insecticides affect non-target species. Our results support the need to study more bee species to more fully understand how different insecticides used in diverse ways affect species at various points throughout their life cycle.

Acknowledgements

We thank members of the MSU CoBRA Lab: G. Sinrud for her help analyzing droplet data and experimental setup, C. Donahoo for her assistance in data collection and experimental setup, C. Brown and D. Reis for their help with experimental setup, and C. Preftakes for assistance with aerosol generator troubleshooting and experimental setup. We also thank JWM Leafcutters, Inc. for providing the test bees and T. Helm for providing the bee board laminates. A special thanks to M. Gaffke and R. Boss for allowing us to use their property as research sites, and J. Stroud (Adapco) and D. Gaiser (Central Life Sciences) for calibration of the aerosol generator and donation of Zenivex® E20. This research was funded by the Mosquito Research Foundation, the Montana Agriculture Experiment Station, and Montana State University. This material is based on work that is supported by the National Institute of Food and Agriculture, Hatch Multistate Project No. W-3045.

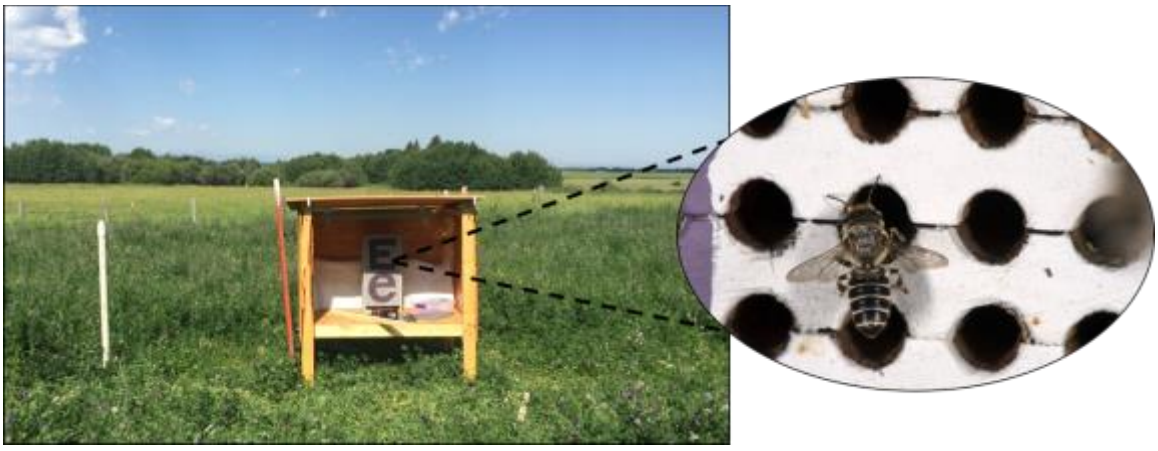

Figure 9. Example of a nest shelter in an alfalfa plot (left) and a *Megachile rotundata* female entering a nest cavity (right). Each shelter consisted of approximately 1,430 cavities and a total of four nest shelters were placed at each location (8 boxes total). Shelters we 134.3 cm x 78.1 cm x 138.1 cm and were approximately 0.61 m off the ground.

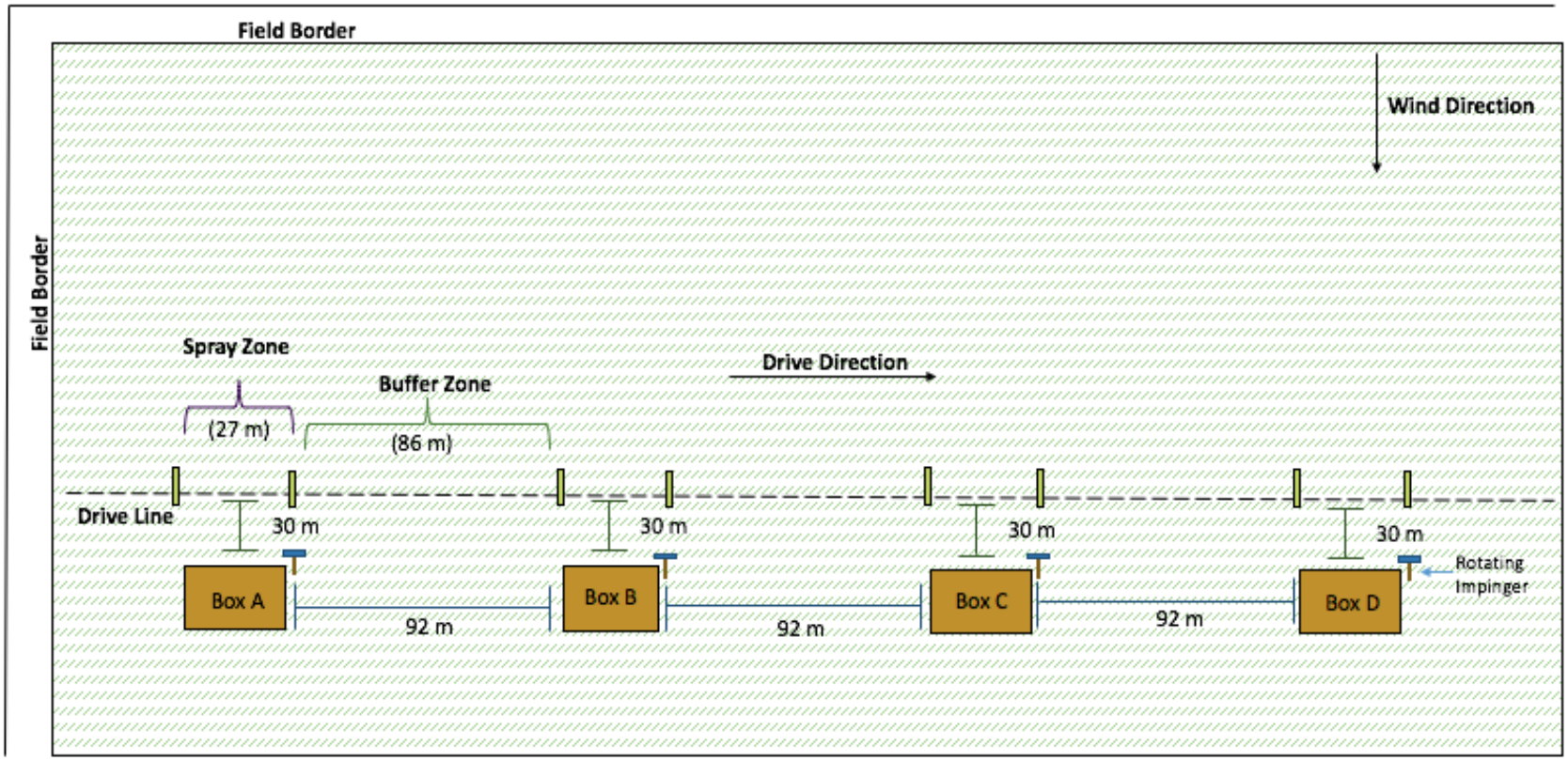

Figure 10. Plot design for an application of Zenivex® E20 (etofenprox) in an alfalfa field.
The field plot was 488 m in length and shelters were set far enough apart to utilize the entire length of the field and to ensure there was enough distance (buffer zone) between each box to account for any insecticide spray drift. For each plot, two boxes were sprayed with Zenivex® E20 at 0.003 kg/ha at a distance of 23 m from each box and two boxes did not receive any treatment.

Table 3. Mean droplet density (± SE) of Zenivex® E20 (etofenprox) and abundance near each insecticide-treated nest shelter. Volume median diameter (VMD) of the droplets, the number of droplets collected, and the relative span factor (RSF), indicating the uniformity of the drop size distribution, are recorded for each pair of slides.

Location	**VMD (µm) (SE)**	**Droplets collected (SE)**	**RSF (SE)**
Field 1	9.01 (0.73)	126.81 (44.21)	0.87 (0.09)
Field 2	9.89 (0.62)	147.66 (37.94)	0.91 (0.06)

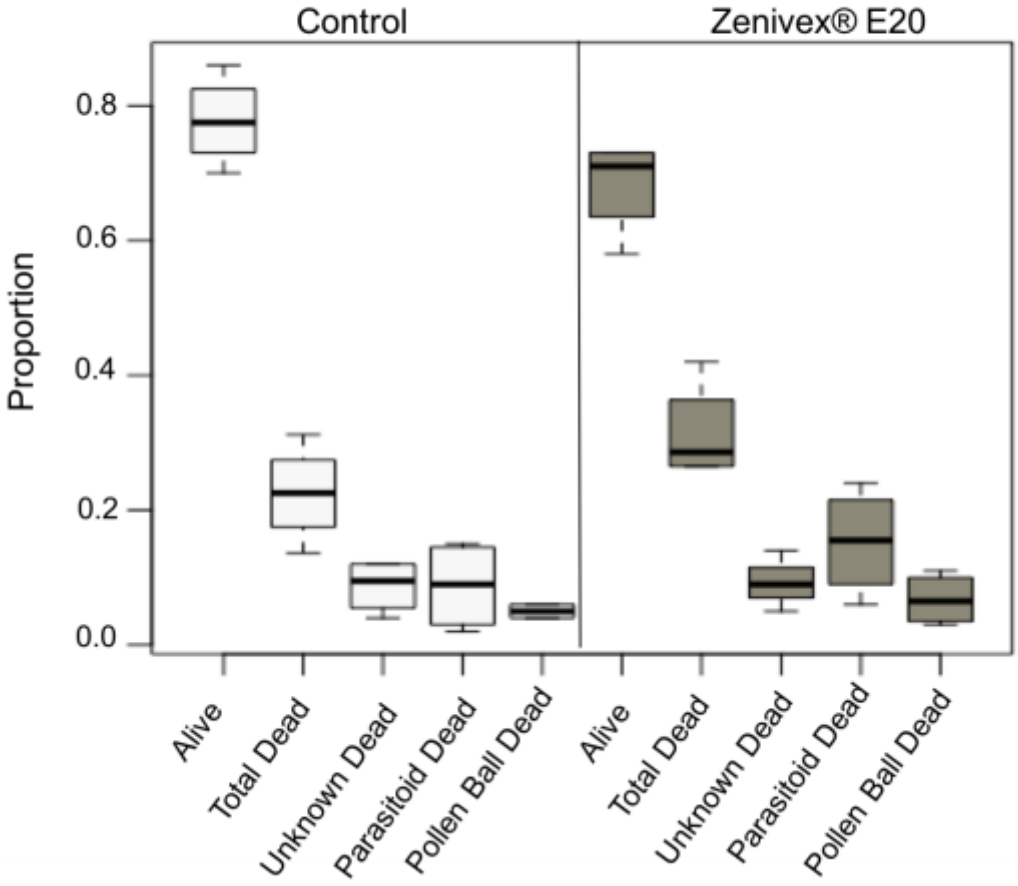

Figure 11. Proportion of individuals present in each category for control (white) and treated (gray) (Zenivex® E20, etofenprox) groups from the total number of individuals sampled. Larvae were characterized as alive and dead (total dead). Dead larvae were categorized as "unknown dead" (dead larva, but cause unknown, "parasitoid dead" death caused by parasitism, and "pollen ball dead" (presence of a pollen ball, but no evidence of larval feeding).

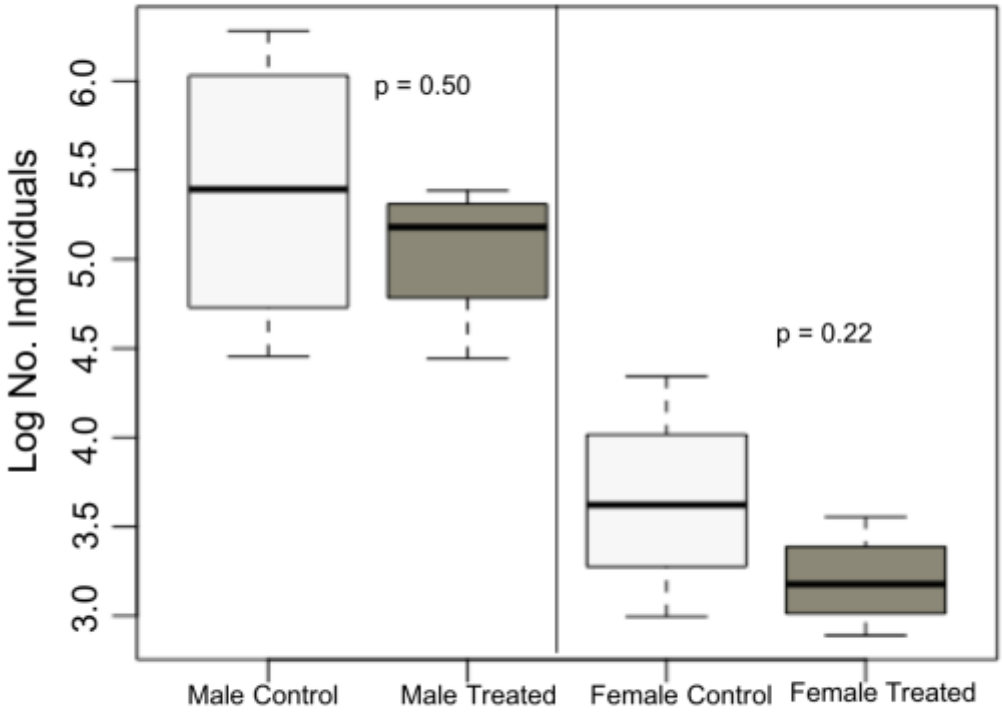

Figure 12. Log number of adults reared from shelters exposed to Zenivex® E20 (gray) and the control group (white) from the previous growing season.

Table 4. Cumulative number of completed nest cavities by female *Megachile rotundata* in 2016.

Time of Application	Treatment		df	P-value
	Control	Zenivex® E20		
	Total No. of Completed Cavities (SEM)	Total No. of Completed Cavities (SEM)		
Before application of Zenivex® E20	455.5 (58.90)	480.0 (33.54)	4	0.73
After application of Zenivex® E20	713.5 (66.26)	802.5 (24.58)	4	0.28

CHAPTER FIVE

CONCLUSION

To have a more comprehensive understanding of how insecticides affect pollinator insect species, studies on insecticide exposure should focus on a variety of different species because life history, routes of exposure, and toxicity because they can vary among species. Most of the information currently available on the effect of insecticides to non-target insects focuses specifically on *A. mellifera* because of its important economic role as a pollinator for many desired and essential products in modern agriculture.

This study provided previously unknown toxicity, exposure, and risk information about how mosquito management insecticides impact *M. rotundata*. We focused on this one species because it is arguably the world's most intensively managed solitary bee and an essential pollinator of alfalfa in the western U.S. In Objective 1, we characterized the toxicity of three pyrethroid insecticides by estimating the LD_{50} of each active ingredient and compared that information to previously existing LD_{50} values for *A. mellifera*. Interestingly, although female *M. rotundata* are about three times smaller than female *A. mellifera*, *M. rotundata* females were generally less susceptible to these pyrethroids, except for deltamethrin, which had a similar LD_{50} endpoint for both species. For this study, we also dosed female *M. rotundata* with the LD_{50} of each active ingredient and measured the difference in respiration over time. Although etofenprox and permethrin had similar LD_{50} endpoints in female *M. rotundata*, the respiration rates after dosing of

these insecticides were significantly different, indicating that these pyrethroids may affect the respiration in different ways. It also indicates that the manner in which *A. mellifera* and *M. rotundata* physiologically respond to topical doses of these insecticides could vary depending on the active ingredient.

Objective 2 of this project involved comparing mortality of newly emerged *A. mellifera* and *M. rotundata* adults to alfalfa foliage that had pyrethroid residues on it after spraying over the alfalfa field via an ultra-low-volume aerosol generator. The purpose of this assay was to better understand bee mortality if they were directly in contact with pyrethroid deposition on leaf surfaces. Because pyrethroids have a relatively low half-life in the environment and ULV droplets are characterized as being extremely small, we hypothesized that no significant mortality would be observed for either of the test species. Our results supported our hypothesis and indicated that when these insecticides are applied over an alfalfa field at dusk, even at the maximum labeled rate, the risk is most likely low to these two bee species.

The final study of this project, Objective 3, primarily focused on adult and larval responses to a single application of a pyrethroid (etofenprox) that was applied at the half-maximum labeled rate directly to active nests of *M. rotundata*. By assessing how this method of application of pyrethroids affects nests of *M. rotundata* when directly targeted, we provided further evidence that spraying these insecticides during hours of low bee activity did not cause significant mortality for this species.

Objective 2 and Objective 3 both represent a reasonable worst case scenario due to both the rate of formulated insecticide applied and the specific targets of insecticide

applications. In a typical setting, applicators would attempt to avoid *M. rotundata* nests by turning off applicators when near them, ultimately resulting in relatively no droplets impinging on nest openings. It is also common practice to avoid application of insecticides during peak floral bloom to mitigate insecticide contamination in floral resources. Because both field studies occurred under these scenarios, we assume that the risk of adverse effects from these insecticides could be even lower by implementing more real-world management practices. However, although our results suggest that pyrethroids may pose low risk to *M. rotundata* when they are both directly and indirectly exposed to the insecticide, further research should focus on more detailed studies throughout the life cycle of this species. For example, we only examined the response of female *M. rotundata*; males have not been examined. Although males do not provide and parental care to the offspring or beneficial courtship to the female, their sperm adds genetic diversity to the population and is essential. More detailed observations of female activity immediately following an insecticide application could also contribute to how females are affected. We only recorded mortality the days following an insecticide application, but perhaps details on the quantity of provisioning flights, ability to locate nests after a flight, and overall productivity could be measured. Although we compared numbers of completed nest cavities and at the end of a season, these other research topics could be informative to producers who may need to apply insecticide multiple times throughout the growing season.

The combination of these studies aids in gaining a more complete understanding of toxicity, exposure, and risk to non-target organisms while also elucidating previously

unknown aspects of solitary bee response to pyrethroid insecticides. Although pyrethroids are highly toxic to bee species, they are a class of insecticides that is commonly used for managing adult mosquito populations and are likely to continue to be used in agricultural and residential settings to manage insect pest populations. By having a more comprehensive understanding of how these insecticides affect bee species, we can use this information to better our management practices and ultimately optimize exposure to pest species while reducing exposure to beneficial species.

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